The Effects and Genetic Mechanisms of Bacterial Species Interactions on Biofilm Formation

by

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Dedicated To The Ones I Love

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Abstract

Bacteria can increase their survival in stressed environments by forming sessile biofilms on surfaces. Natural ecosystems are usually occupied by multiple species, which may interact with and therefore affect biofilm formation of an incoming species. This dissertation research explores the effects of species interactions and investigates genetic mechanisms of species interactions between an environmental strain *Stenotrophomonas maltophilia* and a water quality indicator species *Escherichia coli* on biofilm formation of *E. coli*. It was found that *E. coli* biofilm development was promoted in dynamic flow systems, but inhibited in static batch plates in mixed species culture compared with pure culture conditions. The opposite effects of co-culture on *E. coli* biofilm formation suggested that species interactions may have different impacts under different culture conditions. To enable the mechanistic study of species interactions, a separation method was developed to allow transcriptome analysis of mixed species communities. Transcriptomic responses of *E. coli* to *S. maltophilia* were analyzed to investigate genetic mechanisms of inhibited *E. coli* biofilm formation in static co-culture. Eighty-nine and 108 genes exhibited genetic responses of *E. coli* to *S. maltophilia* co-cultured in biofilm and suspensions, respectively. Several genes were involved with inhibited biofilm formation of *E. coli* in static co-culture. One highly up-regulated gene, *fliA*, was selected for a mechanistic study. It was found that the production of a major monomer of curli, CsgA, as well as cell aggregation were greatly repressed in *E. coli* with *fliA* overexpression. Knocking out *fliA* partially restored the inhibitive effect of co-culture on *E. coli* biofilm growth. Therefore, it was concluded that inhibited *E. coli* biofilm formation by interactions with *S. maltophilia* partially was caused by the induction of gene *fliA* to suppress curli production. Overall, this dissertation examined the effects of species interactions on biofilm formation of *E. coli*, highlighted the impact of environmental conditions on the effect, and revealed partial understanding of species interactions at a genetic level. This fundamental study contributes to understanding of

biofilm formation in real environments with mixed species, and serves as a starting point towards the development of bacteriotherapy for pathogen control using indigenous species for environmental health.

Chapter 1
Introduction

1.1 Background

A biofilm is an aggregation of microorganisms embedded in an extracellular matrix of polymeric substances attached onto a surface. Bacterial biofilms ubiquitously grow almost everywhere where surfaces are in contact with fluids [1-4]. Persistence of biofilms may cause many problems, including in locations where they can cause serious health concerns. Cells in biofilms usually exhibit higher resistance to antimicrobial treatment [5-8]. Pathogenic species become more difficult to control once biofilms are formed and thus cause many recurring and chronic infections [8]. Non-pathogenic biofilms may also be a health concern, due to the potential to harbor pathogens in biofilm matrices and protect them from antimicrobial treatment. For example, many water-borne disease outbreaks are linked to persistent biofilms on internal pipe surfaces in drinking water distribution systems [9]. On the other hand, biofilms may prevent the deposition of invading pathogens in biofilms and serve beneficial roles in preventing infections. For example, biofilms in gastrointestinal tracts contribute to the protection of hosts against pathogenic microorganisms [10]. Thus, persistent indigenous biofilms may be a double-edged sword with regard to incoming pathogens and health concerns, since whether a pathogen could be embedded in biofilms and then reproduce is critical for its survival and functions.

Natural biofilms in medical, industrial, and environmental systems usually consist of various bacterial species [11]. Interactions of these species with an incoming species affect biofilm formation of this species as well as the overall biofilm composition. Understanding the effects of species interactions on biofilm formation and the mechanisms of species interactions will help to better regulate biofilm formation of species of interest. Future applications of such studies include the effective prevention or

cure of harmful species with indigenous microbiota. A successful case of bacteriotherapy for human health was recently reported [12].

The purpose of this study was to explore the effects of species interaction on biofilm formation and to investigate genetic mechanisms of species interactions with regard to biofilm formation. A dual-species model consisting of *Escherichia coli* and *Stenotrophomonas maltophilia* was selected in this study. *E. coli* is an important indicator of fecal contamination in environmental samples, especially in water resources. Positive detection of *E. coli* usually suggests fecal contamination and the presence of pathogenic microorganisms [13]. Some *E. coli* strains themselves are pathogens, resulting in gastrointestinal disease and urinary tract infections. *E. coli* was also selected in part because of the broad knowledge of its biofilm formation and gene regulations. *S. maltophilia* is a widespread bacterial species in the environment. It is frequently isolated from water, soil, sludge, and more recently from various nosocomial systems [14]. Isolation of *E. coli* and *S. maltophilia* from water filtration and distribution systems, rhizosphere, and urinary tract infections showed that they may share the same niches in nature [15-17], where interactions between the two species may take place. Studying this dual-species model can contribute to the understanding of species interactions and biofilm formation of species of interest in a more complex community with multiple environmental species.

This dissertation consists of seven chapters. This first chapter provides the general motivation and background of the study and describes the basic structure of the dissertation. Chapter 2 is a literature review of bacterial species interactions in mixed species biofilms. Chapter 3-6 each presents an independent study written in a manuscript format, which is submitted or prepared for publication. A brief introduction with a literature review and the objectives of each study are presented in each of these chapters. Chapter 7 presents the conclusions from this dissertation research and provides directions for future study. The appendix lists a co-authored study about identification of biofilm regulators using combined computational and experimental methods. It was published in *PLoS One.*

Specifically, Chapter 2 summarizes different effects of species interactions on biofilm formation of species including but not limited to *E. coli* and *S. maltophilia*. Moreover, mechanisms of species interactions, especially genetic mechanistic studies, were reviewed and summarized in this chapter.

Chapter 3 explores the effects of species interactions between *E. coli* and *S. maltophilia* on biofilm formation of *E. coli.* Furthermore, the impact of culture conditions on species interaction with regard to biofilm development was examined. Biofilm cultures were conducted in two laboratory culture systems, static batch in microtiter plates and dynamic culture in flow cells, respectively, simulating environmental conditions with standstill and running water/liquid over the surfaces on which biofilms grow. An explanation of the observed effects on *E. coli* biofilm growth was also briefly explored.

Chapter 4 describes the development and testing of a separation method to allow transcriptome analysis of a mixed species community. This method was developed in order to study genetic mechanisms of species interactions with a focus on *E. coli.* Separation efficiency and preservation of the transcription profile of *E. coli* during separation are presented in details. This study was submitted to *BMC Microbiology* for consideration of publication.

Chapter 5 is the study of genetic responses of *E. coli* to *S. maltophilia* in mixed species cultures. Identification of *E. coli* genes differentially expressed in mixed species suspensions or biofilms compared with those in single species pure cultures are presented in this chapter. Functional analysis and categorization of identified genes show genetic responses of *E. coli* to *S. maltophilia.* Further study includes verification of some identified genes in inhibited biofim formation of *E. coli* in mixed species culture. Gene identification in this chapter brings up research directions for mechanistic studies, of which Chapter 6 is an example.

Finally, Chapter 6 presents a study designed to uncover the mechanisms of gene *fliA* involving in *E. coli* biofilm formation in co-culture with *S. maltophilia*. The gene *fliA* is among the highly induced genes identified in Chapter 5. This chapter firstly inspects the relationship of *fliA* up-regulation with interactions between the two species. The

mechanism is revealed from the study of cell motility, curli production, and biofilm formation with regulated *fliA* expression.

1.2 References

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Chapter 2
Literature Review: Species Interactions in Mixed Species Biofilms

2.1 Abstract

This chapter summarized observations about the effects of species interactions on biofilm formation of various bacterial species from literature studies. The effects were categorized as synergistic, antagonistic, neutral and other influence on biofilm formation. Mechanistic studies of species interactions revealed several mechanisms in different species to pose the effects on other species. This brief review helped to get acquaintance with the current status in the field and provided clues such as study methods and approaches for this dissertation research.

2.2 Introduction

Dual-species biofilm culture in laboratory systems are used in most studies of multiple species, although multiple-species biofilms (e.g. 20 species [1]) and *in vitro* biofilm growth (e.g. in mice [2]) are also explored. A broad review of literature studies on species interactions among various bacterial species including the two model species in this dissertation study, *Escherichia coli* and *Stenotrophomonas,* was conducted. Achieved knowledge about species interactions on biofilm formation was summarized in this chapter.

2.3 Effects of species interactions on biofilm formation

Species interactions are generally categorized as synergistic, antagonistic and neutral effects on biofilm formation by comparing mixed-species biofilms with that of individual single-species biofilms. The object of comparison includes but not limits to biomass, colonization, stability, function, structure and fitness of biofilms.

2.3.1 Synergistic effect

Synergistic effect on biofilm formation was observed among many bacterial species [3-7]. The most obvious phenomenon is a significant increase in biomass of biofilms when two or multiple species were cultured together than cultured individually [5, 8]. Comparison in biomass of biofilms grown in microtiter plates was used to quickly assess the effect of species interactions, especially for screening among many strains [9]. In flowing systems, synergistic effect on biofilm formation was usually observed as stimulated surface colonization of a weak-biofilm-forming strain, possibly due to embedding in biofilm matrix or co-aggregation with a surface-adhesion-proficient strain [5, 10]. Increased cell density of one species in the effluent in co-culture also indicated synergism in biofilm formation in flowing systems, where direct observation of biofilms was difficult to conduct [11]. Assistance from other species in surface colonization could be a critical strategy for the survival and propagation of deficient biofilm-forming species in the environments such as in flowing pipes, where cells in planktonic growth mode are more easily to be removed such as by disinfection [10].

In addition, synergistic interactions were easily observed among species with commensal relationships in substrate usage, such as those demonstrated in biodegradation of organic chemicals [6, 12]. Relying on metabolic product(s) of another species as the only substrate for one species was responsible for the cooperation of the two species and the synergistic effect on biofilm formation. This relationship may drive genetic evolution for an intimate association in biofilms [6]. Improved functions (e.g., degradation efficiency), increased fitness, and enhanced stability of mixed species biofilms also indicate synergistic interactions. For example, mixed-species biofilms displayed a higher efficiency in mercury reduction and remained more stable when encountering a sharp increase in mercury loading [13]. In addition, mixed-species biofilms of four enamel pathogens showed a more powerful function in demineralizing intact enamel than any of the biofilms consisting of only one pathogen [14].

Moreover, increased resistance to antimicrobial reagents, oxidative stress, and bacterial invasion of mixed-species biofilms in comparison with mono-species biofilms also suggests synergistic interactions among species [8, 15, 16]. Protection of sensitive species

by more resistant species was demonstrated within several mixed-species biofilms exposed to disinfectants or antibiotics [1, 17]. The protective effect may increase with the proportion of resistant cells in mixed-species biofilms [1]. A more viscous matrix may be formed due to interactions between different matrix polymers (e.g. exopolysaccharide) produced by different species [18]. Increased viscosity could act as a better barrier to prevent the penetration of antimicrobial reagents into mixed-species biofilms [8, 15]. Spatial organization of different species in biofilms may also contribute to increased resistance. For examples, cells were found to be more intermingled with each other in biofilms when the concentration of toxicant increased [16]. The survival of sensitive species was increased due to its closer distance to the toxicant-degrading species.

2.3.2 Antagonistic effect

Antagonistic effect refers to decreased biofilm formation when one bacterial species is co-cultured with other species. Competition for nutrition is inevitable when several bacterial species occupy the same niche in the environment [19]. Attachable surface is also an important resource specifically for biofilm growth. Species with a higher growth rate and/or stronger surface-attachment ability may have an advantage over co-cultured species when they were competing for limited substrate and solid surfaces [1, 20]. The faster initial occupation of space and rapid propagation of attached cells may result in antagonistic effect on the colonization of other species, or even completely exclude their growth.

Antagonism is most obviously observed when bacteriocin-sensitive species is co-cultured with bacteriocin-producing species. Co-existence of these species in biofilms was reported in several studies [21, 22], while it was hardly observed in planktonic cultures [23]. Examination of biofilm structure showed exclusive microcolonies of each species on the surface [21]. The specific spatial distribution was probably a strategy to alleviate antagonistic interactions. Biofilm matrix may serve as a diffusion barrier for bacteriocin and allow the co-existence of bacteriocin-sensitive species.

Changing the living environment is another strategy that some species use to compete against the others. For example, *Proteus mirabilis* could successfully induce crystal

formation and out-compete other pathogens co-infecting catheterized urinary tract by raising urinary pH [24]. *Actinomyces naeslundii* failed to maintain in mixed-species biofilms possibly due to the quite acidic environment (pH<5.3) caused by other dominant species such as *Streptococcus mutans* [14].

2.3.3 Other effects

Co-culturing with other species may affect biofilm formation of one species, but the effect is hardly categorized as synergism or antagonism as described earlier. Alteration of biofilm structure is an example of this kind of effect. Biofilm of *Pseudomonas aeruginosa* was able to develop in both single and mixed species culture with *Stenotrophomonas maltophilia.* However, the structure of *P. aeruginosa* changed to filamentous architecture in co-culture due to interactions with *S. maltophilia [25]*.

2.3.4 Neutral effect

No significant difference in biofilm formation of one species in mixed species culture compared to that in single species culture is referred as neutral effect [26]. This is the least interested subject for study. It may indicate the fact that no relationship exists between different species on biofilm growth. On the other hand, it does not exclude the possibility that the effect of species interactions on biofilm formation is lower than the detection limit of current methods such as microtiter assay used in many studies [8, 9].

2.4 Mechanisms of species interactions in biofilms

Research into the exploration of mechanisms of species interactions on biofilm formation has been widely conducted to date. Knowledge of the mechanisms not only facilitates the understanding of microbial ecology in real environments, but also contributes to a better management of biofilm formation of species of interest necessarily in future.

Diffusible signal based species interaction was among the best-studied mechanisms. Various signals were secreted by different bacterial species to affect and influence biofilm formation of other species in co-culture. The character, production, and response of different signals by various species were reviewed in details. Non-signal driven interactions including physical cell-to-cell contact, altered surface charge, nutrient

competition, and conjugation etc. are also reviewed. Finally presented in this chapter is the investigation of responses of one species to species interactions from a genetic level and resulted better understanding of genetic mechanisms of species interactions in several most recent studies.

2.4.1 Species interactions via diffusible signals

Quorum sensing signals were produced by many bacterial species, such as *Vibrio fischeri* and *Pseudomonas aeruginosa*, to sense and control their own population densities, as well as their own biofilm formation and expression of a few genes such as those encoding virulence factors [27-31]. Characterized signals in quorum sensing include a series of acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2), etc.

Since many bacterial species produce the same or similar quorum sensing signals, it is not surprising to find that one species could detect and respond to signals produced by another species when they are cultured together. For example, AHLs were found to mediate species communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed-species biofilms both in flow chambers and in lung tissue of infected mice [2]. However, the communication was only unidirectional. Specifically, *B. cepacia* was able to perceive AHLs from *P.aeruginosa*, but not *vice versa*. This was due to the specific composition of AHLs and different sensitivity of AHLs-binding R-homologues of the two species [2]. Another category of quorum sensing signals, the AI-2 signals, was also confirmed to drive a two-side communication between *Escherichia coli* and *Vibrio harveyi* in mixed specie culture [32]. Interestingly, AI-2 signals produced by the two species were actually different in structures [33]. The ability of intra-species communication implied that AI-2 signals released from one species could be converted into the specific form recognizable by the other species [32]. Similar quorum sensing signals based species interactions were also observed among many other species in several studies [34-36].

Another category of signals is growth inhibitors, of which best studied were antimicrobial reagents such as antibiotics. Inhibitory signals produced by one species usually cause antagonistic effects or completely exclude the biofilm formation of other species in co-

culture. For example, an antibacterial protein AlpP produced by *Pseudoalteromonas tunicata* was able to remove other strains that were sensitive to AlpP from mixed-species biofilms [37].

In contrast to inhibitory signals, some signals produced by one species are able to promote biofilm formation of other species. For example, the extracellular molecule indole secreted by *Escherichia coli* was able to stimulate biofilm formation of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* [38]. Some species were confirmed to induce biofilm formation of other species via signals, but the signals were not well characterized. For example, an extracellular signal secreted by *Lactobacillus casei* was responsible for increased biofilm formation of *Saccharomyces cerevisiae* in co-culture [39]. But the signal itself was unknown except its weight of 3 to 5 kDa and its sensitivity to heat.

Considering the wide distribution of signal-producing species and possible inhibitory effects on other species living in the same environment, it is not surprising that many species have developed certain strategies to quench signals and mitigate the effects posed by the signals. For example, quorum-quenching enzymes have been identified in several species such as *Agrobacterium tumefaciens* [40-42]. Co-existence of both signal-producing and signal-inactivating species was identified in mixed-species biofilms in a water treatment system [42]. The mechanism of signal tuning in biofilm formation has been partially approached among some species. For example, the signal indole was converted to an inactivate molecule by *Pseudomonas fluorescens* to enhance *E. coli* biofilm formation in mixed species culture [38].

Spatial distribution of species interacting via signals in biofilms has also been studied. It was found that juxtaposition between different species was required for species communication, especially in an open environment such as a continuous flow system widely used for laboratory biofilm culture [43]. For example, only when the two bacterial species were in very close proximity in biofilms, did *Streptococcus gordonii* respond to extracellular signals produced by *Veillonella atypical* [3]. Position vicinity of two species may be not critical in closed systems such as a batch culture, because signals may

accumulate and reach a threshold level to trigger the response. However, extracellular signals may be able to function between species only over a short distance. Coaggregation of two species may bring two microorganisms closer for interspecies communications via signals [43].

2.4.2 Species interactions with cell-to-cell contact

Coaggregation was discovered between bacterial species isolated from the oral cavity in 1970 [44] and later was observed in many other microbial ecosystems such as freshwater biofilms [45]. Coaggregation brings two species physically in contact and thus help in signal communication [43], metabolic collaboration [3], surface attachment and biofilm formation especially for attachment-deficient species [3, 10, 11, 46].

Coaggregation is usually mediated by adhesins and receptors on aggregated species. Several adhesins have been identified, but with little characterization, in several species [46]. For example, coaggregation of *Fusobacterium nucleatum* with *Porphyromonas gingivalis* was probably mediated by an adhesin of 30-kDa outer-membrane polypeptide [47]. Two adhesin proteins of *Streptococcus gordonii,* SspA and SspB, were found to mediate its coaggregation with *P. gingivalis.* Some adhesins were carried on fimbriae, which were able to penetrate the electrostatic spheres of two bacterial cells and help the adhesin to locate receptors. Less was known about coaggregation receptors. A polysaccharide in cell wall was characterized as a putative receptor [46]. Multiple adhesin-receptors may be involved in a pair of coaggregated species, such as *P. gingivalis* and *S. gordonii* [48]. However, it was found that not all adhesin-receptors were required for biofilm formation of primary colonizers [49].

In contrast, surface proteins may serve as communication signals to prevent other species to adhere with pre-colonized species. For example, it was found that surface protein arginine deiminase (ArcA) of *Streptococcus cristatus* served as a signal to repress the expression of *fim*A in *Porphyromonas gingivalis.* FimA is the major component of fimbriae, which is required to initiate biofilm formation of *P. gingivalis.* As a result, the communication through ArcA excluded *P. gingivalis* in biofilm development with *S. cristatus* [50, 51].

2.4.3 Other species interaction mechanisms

Besides diffusible signals and cell-to-cell contact, some other mechanisms have been revealed. For example, heterogeneous surface charge of *Enterococcus faecalis* was found to stimulate synergistic biofilm formation of *Enterococcus faecalis* with other species such as *Morganella morganii* [52]. In addition, plasmid conjugation was also shown as a driving force in synergistic biofilm formation among several *Escherichia coli* strians [9].

2.4.4 Better understanding of mechanisms from genes involved in species interactions

Identification of genes involved in species interactions has greatly promoted the understanding of mechanisms of species interactions. Individual genes (e.g., *Mfa1* and *wapH*) crucial in species interactions were discovered using methods such as mutant screening [6] and promoter-labeling gene fusions [43]. More genes were identified by cDNA microarray technology to study the transcriptomic response to species interactions [53-56]. Characterization of identified genes has expanded the mechanistic insight. For example, identification of nine genes involved in arginine biosynthesis and transport lead the discovery of stabilized arginine biosynthesis of *Streptococcus gordonii* by co-aggregating with *Actinomyces naeslundii* [53]. Transcriptomic analysis of *Lactococcus lactis* showed dramatic modification when co-cultured with *Staphylococcus aureus*, including ion transport and synthesis [56]. And iron synthesis was confirmed as an interaction pathway between *Pseudomonas aeruginosa* and *S. aureus* [54].

2.5 Implications

Previous studies of mixed species biofilm formation were reviewed. The effects of species interaction on biofilm formation of various bacterial species and mechanisms of species interactions were summarized and discussed briefly. These previous studies provided a strong basis of this dissertation study. It suggested more effective methods and approaches for this type of study in great need, which is one of the focuses of this dissertation study on biofilm formation of *Escherichia coli* and *Stenotrophomonas maltophilia.* Specifically, cDNA microarray technology was explored to investigate the mechanisms of species interactions since it had been proved to be an effective approach.

Furthermore, a separation method was developed to enable the application of cDNA microarray technology in studying mixed species biofilms.

2.6 References

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Chapter 3
The Presence of *Stenotrophomonas maltophilia* Affects *Escherichia coli* Biofilm Formation Differently in Static versus Continuous Flow Conditions

3.1 Abstract

Biofilms in natural environments may harbor pathogenic species or prohibit their growth, depending on the type of species interactions. This study examined the effect of species interactions between *Escherichia coli* and *Stenotrophomonas maltophilia*, representing a pathogen and an environmental strain, respectively, on biofilm formation for two commonly studied conditions, static batch and dynamic flow cultures. *E. coli* biofilm formation was significantly, but differently altered due to interactions with *S. maltophilia* for either culture condition. In a flow cell system, *E. coli* biofilm formation was greatly promoted, possibly due to strengthened surface attachment in the presence of continuous shear force by a *S. maltophilia* biofilm matrix. In contrast, biofilm formation by *E. coli* was reduced in static batch co-cultures, possibly due to nutrient competition, competition for surface attachment, or reduced cell aggregation of *E. coli* cells. Results from this study indicated that the probability of an invading species to successfully colonize pre-existing biofilms not only depended on interactions with existing species but also on environmental conditions, such as the degree of shear force exposure.

3.2 Introduction

Most microorganisms in nature live in sessile biofilms rather than as free-moving planktonic cells. Persistence of biofilms may cause many problems, including health concerns. Cells in biofilms usually exhibit higher resistance to antimicrobial treatment [1-4]. It becomes more difficult to control pathogenic species when they grow in biofilms and, as a result, pathogens in biofilms cause many recurring and chronic infections [4]. While single species biofilms have been widely studied, natural biofilms usually consist of multiple species sharing the same ecological niche [5]. Biofilm formation by one

species could be affected by the presence of other species via mechanisms such as nutrient competition, signal transduction, and coaggregation [6-8]. Indigenous non-pathogenic biofilms may harbor pathogens or may prevent the deposition of other pathogens in biofilms depending on the types of interactions between the pathogenic species and other biofilms species [9, 10]. Studies of species interactions between environmental species and pathogens and the effect of species interactions on biofilm formation will help to understand and control biofilm formation involving pathogenic species.

A dual-species model consisting of *Escherichia coli* and *Stenotrophomonas maltophilia* was selected in this study. *E. coli* is an important indicator of fecal contamination in environmental samples, especially in water resources. Positive detection of *E. coli* usually suggests fecal contamination and the presence of pathogenic microorganisms [11]. Some *E. coli* strains themselves are pathogens, resulting in gastrointestinal disease and urinary tract infections. *S. maltophilia* is a bacterial species found in many different environments. It is frequently isolated from water, soil, sludge, and various hospital systems [12]. Since previous studies have isolated both *E. coli* and *S. maltophilia* from water filtration and distribution systems, rhizosphere environments, and urinary tract infections [13-15], it is possible that they share the same niches in various environments, where interactions between the two species may take place. This study was designed to investigate how biofilm formation was affected by species interactions between *E. coli* and *S. maltophilia* in controlled laboratory systems.

Static cultures in microtiter plates and dynamic cultures in flow cells are the two most widely used systems for laboratory studies of biofilm formation. The two culture conditions can simulate typical environments where biofilms grow relevant for drinking water distribution, including storage tanks and distribution pipes with standstill and running water, respectively. Biofilm formation by one species differs in systems with different fluid characteristics [16]. It is likely that mixed species biofilm formation also varies within different systems. However, most previous studies of species interactions on biofilm formation were performed in only one of these two culture conditions [6, 17].

Therefore, the objective of this study was to study whether culture conditions would affect species interactions on biofilm formation.

3.3 Results

3.3.1 Biofilm formation in flow cell systems

Five parallel flow cell systems (E1, E0.1, S1, E1S1, E0.1S1) were operated. Single species biofilm formation was monitored for *E. coli* (E1 and E0.1, inoculation of 1 ml 10^9 CFU/ml and 10^8 CFU/ml *E. coli* cells, respectively) and *S. maltophilia* (S1, inoculation of 1 ml 10^9 CFU/ml cells). Furthermore, mixed species biofilm formation inoculated with different ratios of the two species (E1S1 and E0.1S1, 1 and 0.1 indicated inoculation of 1 ml 10^9 CFU/ml and 10^8 CFU/ml cells, respectively) was evaluated. Initial attachment of inoculated cells on the glass surface was promoted by pausing the flow of media for one hour. Unattached cells were gradually washed away after the media flow was resumed (time zero) and biofilm formation was then monitored using confocal laser scanning microscopy.

In the system E1 (pure *E. coli* culture), biofilm with cell aggregates was formed at the first monitoring time point (1 h after time zero, simply denoted as 1 h hereafter) (Figure 3.1A), indicating the formation of *E. coli* aggregates and surface attachment during the period of initial attachment. However, most of these initially attached cells were removed after the flow resumed, leaving few cells on the surface (Figure 3.1A, 17 h to 33 h). Small microcolonies were then formed and grew into bigger microcolonies (Figure 3.1A, 33 h to 45 h). Detached cells from microcolonies inoculated other uncovered surface (55 h), and eventually a thick blanket of biofilm covering the entire surface developed after 80 h, with no observable change in biomass density or structure thereafter.

In another single species culture system S1, *S. maltophilia* cells were also able to adhere onto the surface during the stage of initial attachment and formed biofilm by 1 h of flow. At this stage, *S. maltophilia* biofilm consisted of mostly a single layer of attached cells and the amount of attached biomass was much less compared to the amount of *E. coli* biofilm at the same point (system E1, 1 h). However, these initially attached cells were not removed by the media flow and quickly proliferated into a thick biofilm within only

17 h (Figure 3.1A). A portion of *S. maltophilia* cells exhibited a filamentous morphology during this process (Figure 3.1B).

When the same number of *E. coli* and *S. maltophilia* cells were inoculated together into one flow cell system (E1S1), *S. maltophilia* biofilm showed similar growth as in its single species culture (system S1), but thick *E. coli* biofilm formation was much quicker (33 h) compared to that in system E1 (80 h, Figure 3.1A). The biggest difference of *E. coli* biofilm growth in the mixed species culture was that the removal of initially attached *E. coli* cells by the flow as seen in system E1 was not observed in system E1S1. This suggested that the *S. maltophilia* biofilm protected *E. coli* cells from being eliminated by the flow and thus facilitated *E. coli* biofilm formation. This effect became even more apparent in system E0.1S1, which was inoculated with 10-fold less *E. coli* cells. The control system E0.1 only exhibited a few cells near the edge of the flow chamber and no *E. coli* biofilm was detected after four days of operation. In contrast, a thick biofilm of *E. coli* cells gradually developed within two days in system E0.1S1.

3.3.2 Biofilm formation in static batch culture

Different from flow cell systems, static batch cultures in microtiter plates are not exposed to flow during biofilm growth. The effect of species interactions on biofilm formation was further evaluated in this different culture condition.

Biofilm growth in microtiter plates was evaluated by quantifying the amount of biomass of the attached cells after staining with crystal violet. Comparing the amount of biomass of single and mixed species biofilms indicated the effect of species interactions on biofilm formation. Biomass quantification showed that *E. coli* formed much more biofilm than *S. maltophilia,* even when 10-fold less cells were used for inoculation (Figure 3.2A). When the two species were cultured together with different ratios of inoculated cells (0.1:1, 1:1 and 1:0.1), the total amounts of biomass of mixed species biofilms were all significantly less than the biomass of a pure culture *E. coli* biofilm (70% - 90% reduction) (Figure 3.2A).

In addition to biomass quantification, biofilms in static culture were also inspected microscopically to evaluate their structure and composition, especially for mixed species

biofilms. Microscopic images of the biofilms (Figure 3.2B,C,D) confirmed the biomass quantification data in Figure 3.2A. *E. coli* biofilm consisted of multiple layers of attached cells while *S. maltophilia* biofilm only had a single layer of attached cells. Mixed-species biofilms were composed of a single layer of *S. maltophilia* cells interspersed with small microcolonies of *E. coli* cells. These images, as well as quantified biomass, revealed that *E. coli* biofilm formation was significantly reduced in mixed species cultures while biofilm development of *S. maltophilia* was not affected. These results suggested that interactions between *S. maltophilia* and *E. coli* showed inhibitive effects on biofilm formation of *E. coli* but not of *S. maltophilia* in static batch cultures. Significant inhibition was observed even when 10-fold less *S. maltophilia* cells were co-inoculated with *E. coli* (Figure 3.2A).

3.3.3 Effect of diffusible signals on biofilm formation

Previous studies showed that diffusible signal factors (DSF) secreted by *S. maltophilia* could alter biofilm formation of other species [6]. Therefore, experiments were designed to evalute whether the presence of DSF inhibited *E. coli* biofilm formation in static co-cultures.

Since supernatant of cell cultures contains DSF, supernatant samples were acquired from planktonic cultures of *S. maltophilia* harvested during exponential and stationary phases. The effect of DSF on *E. coli* biofilm formation was evaluted by supplementing growth media with these supernatant samples. The results indicated that neither of the two supernatant samples had an effect on biofilm formation of *E. coli* (Figure 3.3A).

In a second experiment to evalute the effect of DSF, transwell systems were used for cell contact-free co-culture between the two species, which had the advantage over the previous experiment that DSF was available during any growth phase. *E. coli* biofilms were present in each well, while *S. maltophilia* cells grew in inserts, which were inserted in each well. Porous membranes at the bottom of each insert allowed DSF to diffuse into the corresponding well, but separated *S. maltophilia* cells from direct contact with *E. coli* cells. A mixed culture of *E. coli* and *S. maltophilia* was also grown in an insert, in case some specific DSF may be produced in mixed species culture conditions. Both cell

contact-free cultures inhibited *E. coli* biofilm formation to a small degree (approximately 15% decrease in biomass) (Figure 3.3B). However, this small amount of inibitions only partially explained the much greater inhibition (70-90% biomass reduction) observed when *E. coli* biofilm was grown in mixed species cultures (Figure 3.2).

3.3.4 Reduced cell aggregation and surface attachment

Since cell-contact between *E. coli* and *S. maltophilia* cells was suggested to be responsible for the inhibition of *E. coli* biofilm formation in static culture, the effect of mixing *S. maltophilia* cells with *E. coli* on *E. coli* cell aggregation (adherence of cells to cells) and surface attachment (adherence of cells onto surface) was examined. The degree of cell aggregation was expressed as the percentage of *E. coli* cells forming aggregates and settling down from the top layer of a static culture column due to gravity. Aggregation of *E. coli* cells was reduced from 99.9% in single species culture to 96.7% and 80.9% when mixed with10-fold less and an equal number of *S. maltophilia* cells, respectively (Figure 3.4A).

Initial surface attachment measured the adhesive property of cells during short-term contact with the surface. The amount of surface-attached cells (shown as biomass) was measured after suspended cells were deposited on the surface for one hour. Results showed that initial attachment of *E. coli* was greatly reduced (85% reduction) when mixed with *S. maltophilia* cells (Figure 3.4B). The strength of surface attachment of *E. coli* was further investigated by conducting an invasion experiment. Pre-developed *E. coli* or *S. maltophilia* biofilm was allowed to be invaded by suspended cells of the other species for 24 h. Microscopic images revealed that *S. maltophilia* cells were able to remove most surface-attached *E. coli* cells and took over the surface (Figure 3.5). In contrast, *E. coli* cells failed to eliminate *S. maltophilia* cells from the surface and hardly adhered onto unoccupied surface or *S. maltophilia* biofilm (Figure 3.5). These results indicated that *E. coli* cells were able to attach onto surface, but the strength of their surface attachment was much weaker than that of *S. maltophilia* cells. As a result, *E. coli* cells were out-competed in surface adherence by *S. maltophilia.* The lower cell aggregation and surface attachment of *E. coli* cells when mixed with *S. maltophilia* cells

may explain the greatly inhibited *E. coli* biofilm formation in mixed species static co-cultures.

3.4 Discussion

Comparing biofilm formation of *E. coli* or *S. maltophilia* in single species cultures with mixed species cultures indicated a strong effect of species interactions on biofilm development. Two laboratory model systems, microtiter plates and flow cells representing static batch and dynamic flow conditions, respectively, were used to culture biofilms in this study. Different culture conditions not only influenced mono-species biofilm formation of *E. coli* or *S. maltophilia*, but also showed an effect on species interactions between *E. coli* and *S. maltophilia* with regard to biofilm formation.

The two culture conditions in microtiter plates and flow cells differed in nutrient supply, hydrodynamic parameters, shear force, accumulation of metabolites, suspended cell growth, etc. These parameters likely affect biofilm development in a species-specific way as indicated by several previous studies that observed different effects of culture conditions on biofilm formation by several species [16, 18-20].

3.4.1 Mono-species biofilm formation varied in static and dynamic cultures

Understanding single species biofilm formation forms the basis to study mixed species biofilm formation and species interactions. Therefore, we first analyzed *E. coli* and *S. maltophilia* mono-species biofilm development for two culture conditions. We observed that *E. coli* formed a thick biofilm after 22 h in static culture as reported previously [21]. However, *E. coli* developed biofilms much slower in flow cells. Specifically, it took more than three days to form a mature biofilm in a flow cell system with a large inoculum (1 ml, 10^9 CFU/ml), while no biofilm had developed four days after inoculation with 10-fold less cells (1 ml, 10^8 CFU/ml). One reason for the slow biofilm development by *E. coli* in flow cells was the detachment of initially adhered cells by continuous flow as observed in Figure 1A. Surface attachment of *E. coli* cells was shown to be weak in flow cells. Similar observations were made during an invasion experiment (Figure 3.5), even though the amount of initially attached *E. coli* cells was high (Figure 3.4B). In static microtiter plates, there was no shear force so cells were not removed once attached.

Moreover, planktonic cells accumulated in microtiter plates may also contribute to cell aggregation and thus biofilm formation since the *E. coli* strain showed high aggregation ability (Figure 3.4A). Planktonic replication was previously reported as essential for biofilm formation of other species [16].

In contrast to *E. coli*, *S. maltophilia* formed little biofilm in microtiter plates (Figure 3.2) but quickly developed mature biofilms in flow cell systems (less than 17 hours after inoculation, Figure 3.1A). Similar characteristics of *S. maltophilia* biofilm formation were also reported in other studies [6, 22]. Although the amount of initially attached cells was low (Figure 3.1A and Figure 3.4B), surface attachment was sufficiently strong to survive continuous flow (Figure 3.1A). A single layer of attached cells was able to develop into thick biofilms in flow cells, but not in microtiter plates. Microscopic images showed that thickening of *S. maltophilia* biofilm started from the formation of a filamentous network in dynamic culture (Figure 3.1B). However, no filamentous cells were observed in static culture even with a longer incubation (72 h). This result may explain why the biofilm did not become thicker in microtiter plates. A previous study showed that the switch to filamentous morphology in dynamic culture was triggered by diffusible signal factors (DSF) consisting of a few fatty acids [6]. However, DSF, if produced, should accumulate in batch cultures more easily than in flow cells. Several possible reasons may explain the absence of filamentous cells in microtiter plates. Firstly, DSF may be not produced in stationary growth phase, while *S. maltophilia* cell growth reached late stationary phase after 22 h culture in microtiter plates. In addition, *S. maltophilia* may secrete other signals or secondary metabolites to inhibit the switch to filamentous cell growth. These signals or metabolites may have accumulated in microtiter plates so that filamentous biofilm were not observed in the batch culture experiments.

In summary, the combination of specific culture conditions and cell properties of *E. coli* and *S. maltophilia* determined their different behavior in biofilm formation. The absence of shear force and high cell aggregation of suspended *E. coli* cells resulted in thick biofilms of *E. coli* in static batch culture. Strong surface attachment and filamentous cell growth promoted biofilm growth of *S. maltophilia* in dynamic culture only.

3.4.2 *E. coli* biofilm formation affected by species interactions

When the two species were cultured together, *S. maltophilia* biofilm formation resembled that observed in pure culture, but *E. coli* biofilm development was altered significantly. Specifically, biofilm formation of *E. coli* was promoted in dynamic conditions (Figure 3.1) but inhibited in static cultures (Figure 3.2) due to interactions with *S. maltophilia*.

The enhanced *E. coli* biofilm formation in flow cells was believed to result from reduced cell detachment due to the presence of the *S. maltophilia* biofilm matrix. Detachment of initially attached *E. coli* cells was the reason for little *E. coli* biofilm growth in pure culture as discussed above. No obvious detachment of *E. coli* cells was observed in mixed species culture (Figure 3.1A). Enhanced surface colonization of *E. coli* by other species in hydrodynamic systems was reported in several studies [19, 23, 24], and one of these studies found that co-aggregation with proficient biofilm forming species was the reason for enhanced surface colonization [23]. However, aggregates of *E. coli* with *S. maltophilia* were not observed in the effluent in this study (data not shown). Shear force caused by fluid flow was the primary drive of cell detachment [25]. It was shown that flow velocity near the surface with biofilm was reduced to 50% compared to that without biofilm [26]. It is reasonable to infer that initially attached *E. coli* cells were exposed to reduced shear force due to the quickly developed biofilm matrix of *S. maltophilia*. As a result, the detachment of *E. coli* cells due to fluid flow was reduced and biofilm formation was promoted as observed in Figure 3.1A. Further studies are needed to explore other unknown mechanisms that may play a role in this observation.

In static batch culture, competition for nutrients, reduced cell aggregation, and competition for surface space by *S. maltophilia* may all contribute to inhibited *E. coli* biofilm formation. Unlike the continuous supply of media in flow cells, nutrient supply is limited in batch cultures so that competition for nutrients is more intense and may affect cell growth of one species. Cell density of *E. coli* was lower, although not significantly, in planktonic co-culture compared to that in pure culture (18 or 33% lower, $p>0.05$ when the ratio of inoculated *E. coli* to *S. maltophilia* was 1:0.1 or 1:1, respectively). Cell aggregation, which was critical for *E. coli* biofilm formation [21], was reduced significantly when mixed with *S. maltophilia* cells (Figure 3.4A). Surface attachment of

E. coli was shown to be weaker than that of *S. maltophilia* as discussed above. Moreover, invasion experiments showed that suspended *S. maltophilia* cells were able to remove pre-developed *E. coli* biofilms (Figure 3.5). It can be inferred that suspended *S. maltophilia* cells grown in microtiter plates could remove surface attached *E. coli* cells and further inhibit *E. coli* biofilm formation besides competition for surface attachment. In contrast to flow cell systems, suspended *S. maltophilia* cells were removed by media flow and thus failed to remove *E. coli* cells embedded in their biofilm matrix.

It remains unclear why *E. coli* cells could not attach onto the thin layer of *S. maltophilia* cells in the static culture as they did in the dynamic culture. In a preliminary experiment, we showed that *S. maltophilia* biofilm killed by UV lost the ability to avert *E. coli* cells from attaching onto *S. maltophilia* cells and, as a result, a thick *E. coli* biofilm developed on the matrix of dead *S. maltophilia* cells (data not shown). Live suspended *S. maltophilia* cells grown and accumulated in microtiter plates may contribute to the prevention of *E. coli* from attaching to the *S. maltophilia* biofilm. In addition, although our studies found that supernatant from *S. maltophilia* cultures failed to inhibit *E. coli* biofilm development, we could not exclude completely the role of DSF and other exudates in the inhibition of *E. coli* biofilm formation in microtiter plates. Further research is necessary to explain these observations, including the use of purified DSF to test its effect on *E. coli* biofilm formation.

3.5 Conclusions

This study showed that biofilm formation of *E. coli* was affected by interactions with another species *S. maltophilia*. Moreover, the effect of species interactions on biofilm formation altered with culture conditions. With regard to *E. coli* biofilm formation, species interactions behaved antagonistic in static batch culture but synergistic in dynamic culture. The fact that species interactions varied among different species was reported in previous studies [17, 24]. However, altered species interactions with culture conditions have not yet been reported.

Microtiter plates and flow cell systems are the two most commonly used laboratory systems in studies of biofilms, representing environments such as water storage towers

and delivery pipes in distribution systems, respectively. Results from this study suggest that the probability of an invading species to successfully colonize pre-existing biofilms not only depends on interactions with existing species but also on environmental conditions, such as shear force due to hydrodynamic parameters. Survival of a species, specifically an incoming pathogen, may be greatly enhanced by embedding in biofilms [4], similar to the increased *E. coli* biofilm formation in the presence of *S. maltophilia* biofilm in flow cells in this study. Controlling biofilm formation and the resulting fate of pathogens may be achieved by altering indigenous biofilm species or by changing hydrodynamic conditions.

This study of *E. coli* and *S. maltophilia* provided an interesting model of the impact of species interactions on biofilm growth. Further investigation of genetic mechanisms of interactions between these two species, for example by identifying and characterizing genes involved, are desired for a better understanding of species interactions and for management of species interactions in controlling pathogens.

3.6 Materials and Methods

3.6.1 Bacterial strains and cultures

E. coli K-12 PHL644/pMP4655-GFP and *S. maltophilia*/pBPF-mCherry were used in this study. *E. coli* and *S. maltophilia* were labeled with a constitutively expressed green fluorescence protein (GFP) and red fluorescence protein mCherry, respectively. The culture media consisted of 10-fold diluted Luria-Bertani broth (0.1×LB), supplemented with 40 μg/ml tetracycline and 20 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) for *E. coli* and *S. maltophilia*, respectively. Planktonic cultures were inoculated from single colonies on LB agar plates and incubated at 30 °C overnight (13 h) with continuous shaking (250 rpm). Cells were pelleted by centrifugation (3,000×g, 3 min), and re-suspend in fresh 0.1×LB to inoculate biofilm culture systems. The cell densities of *E. coli* and *S. maltophilia* were measured with the plate count method and adjusted as needed.

3.6.2 Biofilm culture in flow cell systems

Flow systems were assembled with three-channel glass-bottom flow cells (Stovall, Greensboro, NC) as described previously [27]. Culture medium consisting of 0.1×LB was supplied continuously (0.12 ml/min) with a peristaltic pump. The medium flow was paused for inoculation of 1 ml single species *E. coli* or *S. maltophilia,* or a mixture of the two species with densities adjusted to 10^9 or 10^8 CFU/ml. Inoculated cells were allowed to attach onto the surface for one hour before the medium flow resumed, which corresponded to a time of 0 h. Growth of biofilms was monitored with confocal laser scanning microscopy (see below) every few hours until mature biofilms were developed with no further observable change in biomass or structures. Five flow cell chambers were run in parallel for single or mixed species biofilm culture with different inoculation ratios from the same overnight planktonic cultures (E1, S1, E1S1, E0.1, E0.1S1). The whole flow cell system was set at room temperature (20 °C). Each biofilm experiment in the flow cell system was replicated three times.

3.6.3 Biofilm growth in static batch plates

Biofilms were grown in 96-well Nunclon plates (Fisher Scientific, Pittsburgh, PA) for biomass quantification. Glass-bottom 24-well plates (MatTek, Ashland, MA) were used to culture biofilms for microscopic monitoring and biofilm imaging. Re-suspended cells from overnight planktonic cultures, single or mixed species at ratios of 0.1:1, 1:1, and 1:0.1, were inoculated into 0.1×LB (1:100 by volume) in multi-well plates. The plates were set static for biofilm growth at room temperature (20 °C) for 22 h. Suspended cells in each well were gently removed and washed three times with phosphate buffered saline (PBS, pH 7.2). Biofilm biomass was quantified by staining with crystal violet [28] and the results are expressed as optical density (arbitrary units). Four replicate measurements were conducted for each biofilm and the mean value of biomass was reported. The same biofilm growth in 96-well plate was replicated three times independently and similar biomass was acquired. Biofilms in 24-well plates were immersed in PBS for microscopic imaging after three cycles of wash as described above.

3.6.4 Supernatant preparation and cell contact-free culture

Planktonic cultures of *S. maltophilia* were harvested after 4 h (exponential phase) and 15 h (stationary phase) of growth at room temperature after inoculation. Supernatant was acquired by filtering planktonic cultures through membrane filters (0.22 μm, Millipore, Billerica, MA). *E. coli* biofilms grown in 0.2×LB supplied with equal volume of either of the two supernatants were compared with *E. coli* biofilms grown in 0.1×LB in order to test the effect of supernatant on biofilm formation by *E. coli*.

Cell-contact free cultures were conducted in a 24-well Transwell system (Corning, NY), with an insert made of polycarbonate membrane (0.4 μm) for each well. Bacterial species grown in the insert and in the corresponding well were separated from direct cell-to-cell contact, but allowing secreted signal molecules to diffuse freely in each part of insert and well. *S. maltophilia* or *E. coli*-*S. maltophilia* co-culture was grown in the inserts, while *E. coli* biofilm cultures were grown in the corresponding wells. Culture conditions in Transwells and biofilm biomass quantification were the same as those in 96-well microtiter plates as described above.

3.6.5 Initial attachment assay

Overnight cultures of *E. coli* and *S. maltophilia* were re-suspended in fresh 0.1×LB and adjusted to the same cell density (10^9 CFU/ml). Pure *E. coli, S. maltophilia,* or mixtures of the two species (1:1 ratio) were added into a 96-well plate (100 μl/well) and set static at room temperature (20 °C) for 1 h for initial attachment. Suspended cells were gently removed and washed three times with PBS. Biomass of attached cells showing initial attachment ability was quantified with crystal violet staining as stated above.

3.6.6 Cell aggregation assay

Overnight cultures of *E. coli* and *S. maltophilia* were re-suspended in fresh 0.1×LB. Two mixtures of *E. coli* with 10-fold less and with the same amount of *S. maltophilia* cells were prepared. Pure *E. coli* and the two mixtures were added into 5-ml test tubes (Fisher Scientific, Pittsburgh, PA) (3 ml/tube) to form a culture column and set static on the bench for 24 h. Cells were allowed to adhere together to form aggregates, which

gradually settled down due to gravity. As a result, the cell density in the top layer of the culture column decreased. *E. coli* cell aggregation was reported as the relative decrease in cell density (percentage) due to aggregates formation and settling. *E. coli* cell density in both pure and mixtures was measured by the plate count method using selective agar. The experiments were repeated independently twice and showed similar results.

3.6.7 Biofilm invasion assay

Pure *E. coli* or *S. maltophilia* biofilms were developed in microtiter plates for 24 h as described above. Suspended cells were gently removed and washed three times with PBS. Re-suspended *E. coli* or *S. maltophilia* cells from overnight planktonic cultures were added (100 μl/well) into each well to invade pre-formed *S. maltophilia* and *E. coli* biofilms for another 24 h, respectively. Suspended cell were then gently removed, washed three times with PBS. Biofilms before and after invasion were imaged with fluorescence microscopy (Olympus, Wirtz, VA). The same invasion experiments were replicated three times with similar results.

3.6.8 Imaging biofilms

Imaging biofilms cultured in flow cell systems and in microtiter plates were acquired with a confocal laser scanning microscope (Fluoview^{TM}, Olympus, Wirtz, VA) with filter sets for monitoring GFP and mCherry fluorescence for *E. coli* and *S. maltophilia*, respectively. Images were obtained randomly from three to six spots in the center of each flow chamber or each well of the microtiter plates. Biofilms grown near the edge of a flow chamber were acquired only if no biofilms were observed in the center of a flow chamber for pure *E. coli* culture with low inoculation (system E0.1). Three-dimensional images were reconstructed using the software Volocity 3.2 (Improvision Inc., Waltham, MA) from a stack of confocal microscopy images of x-y sections of biofilm samples.

3.7 Figures

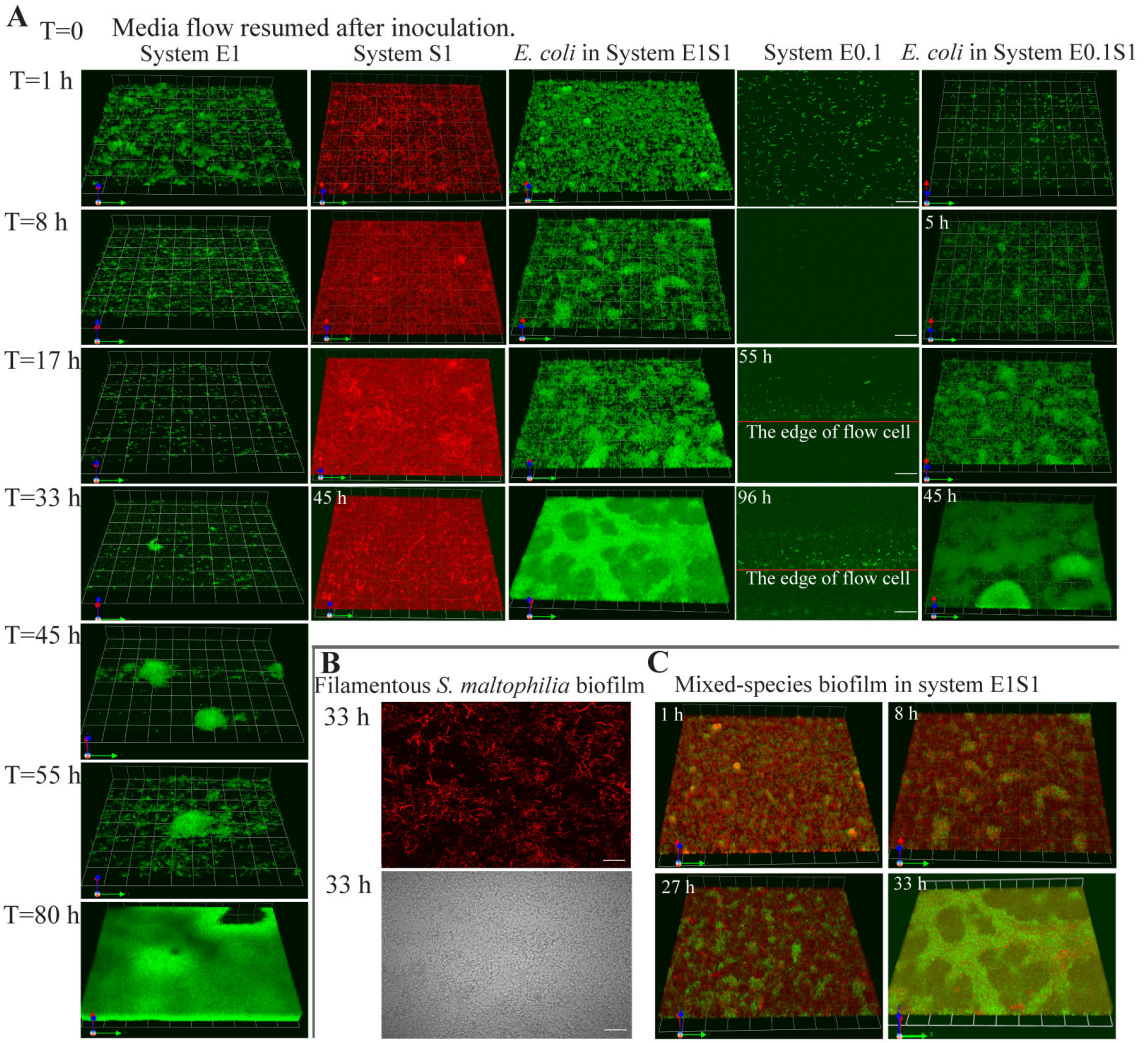

Figure 3.1 Biofilm development in flow cell systems

(A) Images showing the process of biofilm development in flow cell systems, from left to right, pure *E. coli* biofilm (E1), pure *S. maltophilia* biofilm (S1), *E. coli* in mixed species biofilm (E1S1), pure *E. coli* biofilm (E0.1), and *E. coli* in mixed species biofilm (E0.1S1). The numbers 1 and 0.1 correspond to inoculation of 1 ml 10^9 CFU/ml and 10-fold less 10^8 CFU/ml cells, respectively. *E. coli* (carrying pMP4655-GFP) and *S. maltophilia* (carrying pBPF-mCherry) are shown as green and red cells, respectively. Images in the same row were taken at the same time after medium flow resumed unless specifically labeled. Red lines in two images of system E0.1 (55 h and 96 h) show the edge of flow chambers. All other images were randomly taken over spots in the center of the flow chambers. Embedded images in (B) show fluorescence and bright field microscopic images of filamentous *S. maltophilia* biofilm developed in system S1 at 33 h and in (C) show combined two-color mixed species biofilms grown in system E1S1. Grid size is 26.7 μm and scale bar is 20 μm.

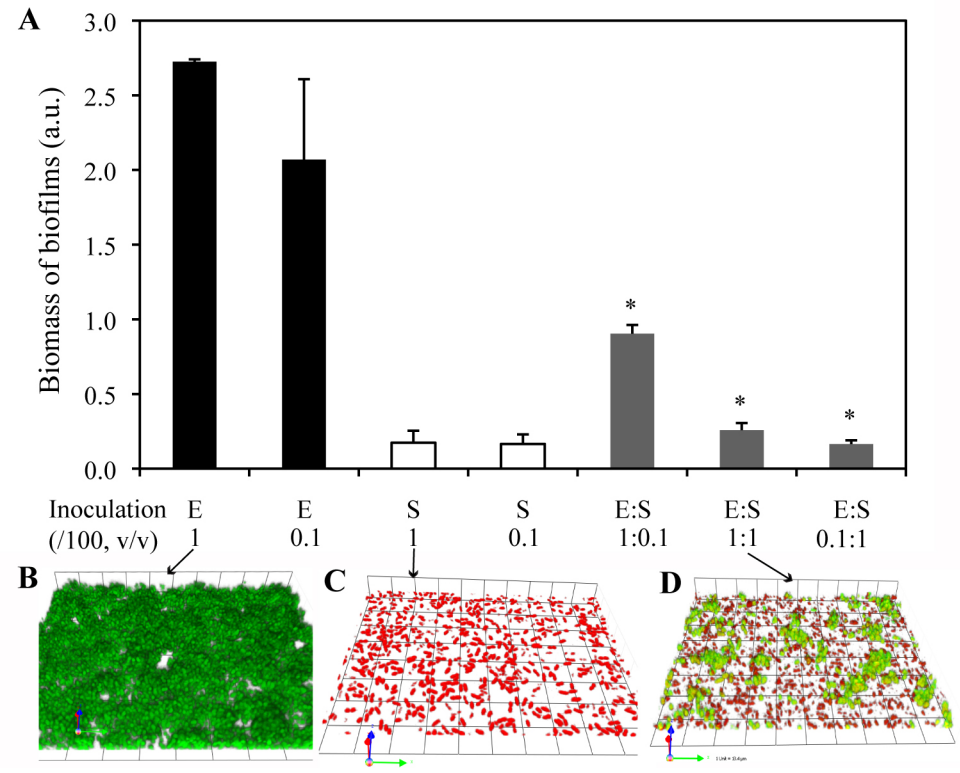

Figure 3.2 Biomass and 3-D images of biofilms in static batch culture

(A) Quantified biomass of single and mixed species biofilms and re-constructed 3-D biofilm images of *E. coli* biofilm (B), *S. maltophilia* biofilm (C), and mixed-species *E. coli* and *S. maltophilia* biofilm (D) formed on glass-bottom microtiter plates in static culture. E stands for *E. coli*/pMP4655-GFP, shown as green cells in images, S indicates *S. maltophilia*/pBPF-mCherry, shown as red cells in images, and E:S stands for *E. coli* and *S. maltophilia* mixed species cultures. Numbers 1 and 0.1 stand for inoculation of, respectively, 1 μl and 0.1 μl overnight cultures (5×10^8 CFU/ml) into 100 μl broth. Biomass quantification was measured by staining biofilm cells with crystal violet. Three independent cultures were performed, each with four replicates. Results from one independent culture are shown in (A). Error bars are standard deviations of three to four replicates. Symbol * indicates significant decrease ($p<0.01$) compared to pure *E. coli* biofilm. Arrows indicate the same culture conditions in which biofilms were grown for biomass quantification and biofilm imaging. 3-D images were re-constructed from a series of confocal laser scanning images taken randomly over the spot near the center of each well in microtiter plates.

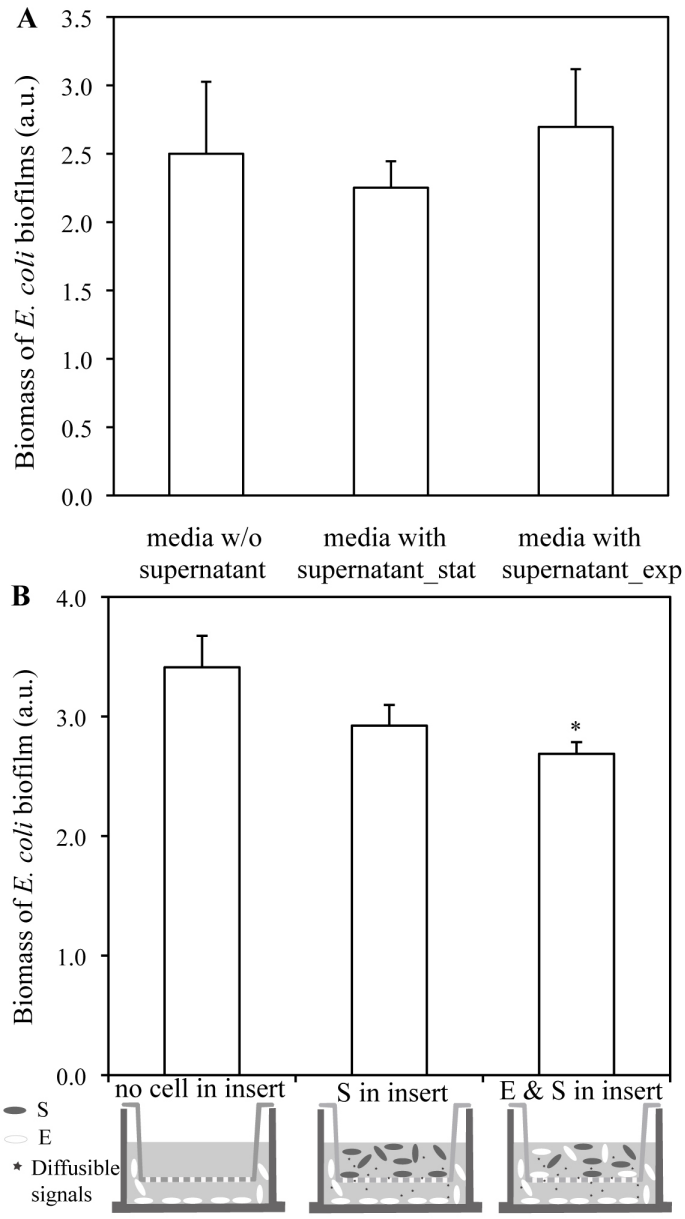

Figure 3.3 Effect of supernatant and cell contact-free culture on *E. coli* biofilm formation

Biomass of *E. coli* biofilms cultured (A) in media supplemented with supernatant from *S. maltophilia* cultures harvested during stationary (stat) or exponential (exp) growth phase, and (B) in Transwell systems with *S. maltophilia* (S) or *E. coli* and *S. maltophilia* (E&S) cells in the insert. Transwell systems corresponding to each culture condition in (B) are shown with white cells standing for *E. coli,* grey cells for *S. maltophilia* and stars as diffusible signals. Biomass was quantified by staining biofilm cells with crystal violet. Each culture was conducted independently twice, each with three/four replicates. Error bars are standard deviations of at least three replicates. Symbol * indicates significant difference ($p<0.05$) from the control of *E. coli* biofilm formation in the Transwell system with no inoculation in the insert.

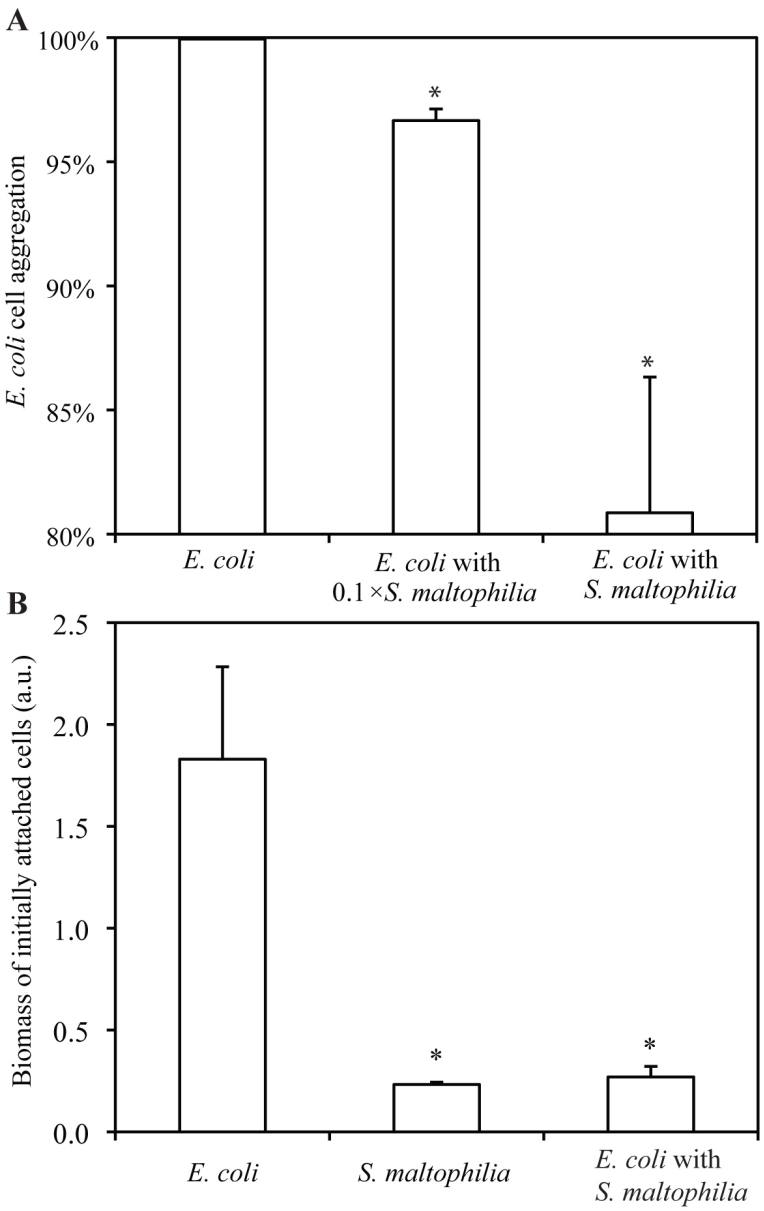

Figure 3.4 Effect of mixing with *S. maltophilia* cells on *E. coli* cell aggregation and initial attachment

The effect on (A) *E. coli* cell aggregation and (B) initial attachment due to mixing with *S. maltophilia* cells. *E. coli* cell aggregation is shown as the percentage of *E. coli* cells that formed aggregates and settled down from the top layer of a culture column due to gravity after being static for 24 h. *E. coli* cell density in the top layer was quantified with the plate count method using selective agar. "*E. coli* with 0.1× *S. maltophilia"* and "*E. coli with S. maltophilia"* indicate, respectively, 10-fold less and equal number of *S. maltophilia* cells were mixed with *E. coli.* Similar results were acquired from two independent replicates. Error bars show the standard deviations in density counting. Initial attachment measured the amount of cells attached to the surface in 1 h. Error bars are standard deviations of three replicates. Symbol * indicates a significantly different result ($p<0.05$) from the control of pure *E. coli*.

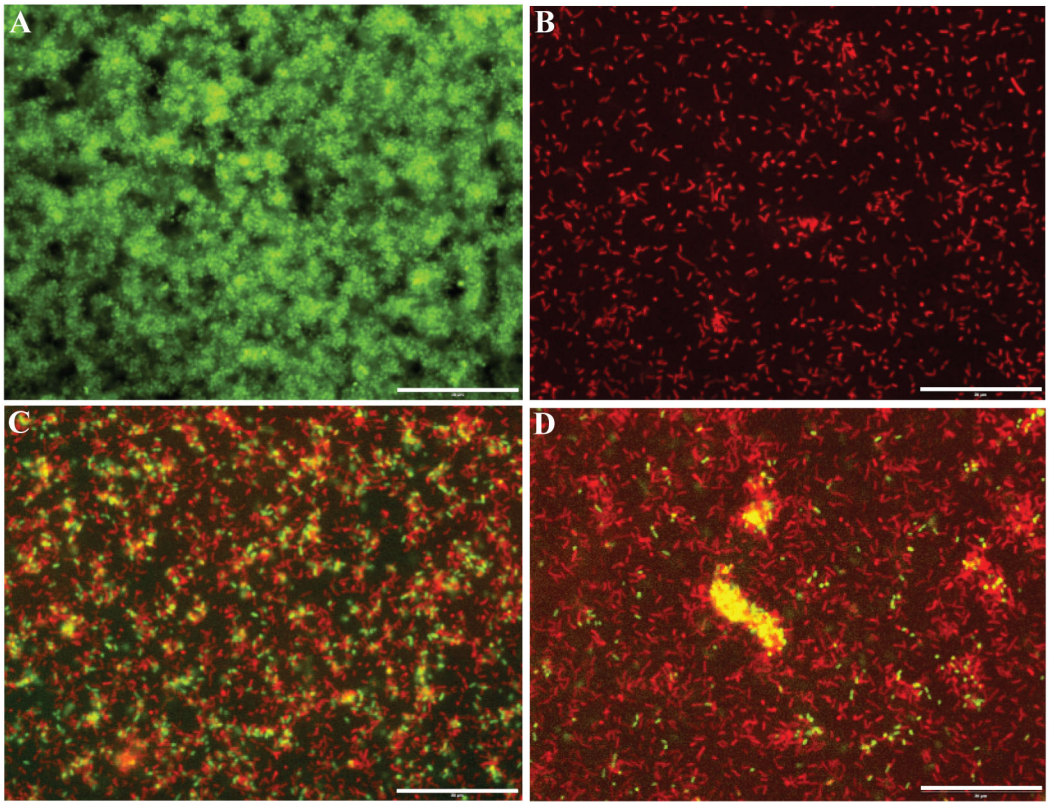

Figure 3.5 Invasion of pre-developed biofilms by suspended cells of the other species

Fluorescence microscopy images of pre-developed biofilms of (A) *E. coli* and (B) *S. maltophilia* and of biofilms after invasion by (C) *S. maltophilia* and (D) *E. coli* cells. *E. coli*/pMP4655-GFP cells are shown as green or yellow and *S. maltophilia*/pBPF-mCherry as red cells. Pre-developed biofilms were cultured in microtiter plates for 24 h at room temperature. Invaded cells were 1 ml suspended cells from overnight planktonic culture (10^9 CFU/ml). Scale bar is 20 μm.

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Chapter 4
Separation of *Escherichia coli* K-12 from Mixed Species Communities for Transcriptome Analysis

4.1 Abstract

Study of bacterial species interactions in a mixed species community can be facilitated by transcriptome analysis of one species in the community using cDNA microarray technology. However, current applications of microarrays are mostly limited to single species. The purpose of this study is to develop a method to separate one species, such as *Escherichia coli*, from communities for transcriptome analysis. *E. coli* cells were separated from dual-species (*E. coli* and *Stenotrophomonas maltophilia*) communities using immuno-magnetic separation (IMS). High recovery rates of *E. coli* were achieved. The purity of *E. coli* cells was as high as 95.0% separated from suspended mixtures consisting of 1.1 – 71.3% *E. coli,* and as high as 96.0% separated from biofilms with 8.1% *E. coli* cells. Biofilms were pre-dispersed into single-cell suspensions. Reagent RNA*later* was used during biofilm dispersion and IMS to preserve the transcriptome of *E. coli*. Microarray study confirmed that very few *E. coli* genes (8 out of 4,289 ORFs) exhibited a significant change in expression during dispersion and separation, indicating transcriptional profiles were well preserved. The method based on immuno-magnetic separation (IMS) and use of RNA*later* was developed to separate *E. coli* cells with preserved transcriptome from mixed species communities. The method combined with cDNA microarray should be very useful to study species interactions in mixed species communities.

4.2 Introduction

Microorganisms in natural environments rarely grow as single species, but grow as mixed species consortia in which a variety of intra- and inter-species interactions take place [1, 2]. Previous studies have shown that species interactions play an important role in the

development, composition, structure, and function of microbial consortia in biofilms as well as in suspended growth communities [3-5]. Studies of species interactions have promoted the understanding of microbial activities in mixed species communities [6-8].

Identification of relevant genes is an important step toward the elucidation of the molecular mechanisms of species communication. cDNA microarray technology has been widely used for mono-species cultures, but only a few for mixed species consortia due to broad cross hybridization among species [6, 9, 10]. Variable conservation of genes existed across bacterial species [11]. Non-target transcripts have been shown to cross hybridize in oligonucleotide microarray studies [12]. The problem was addressed previously by carefully selecting co-cultures of a gram-negative and a gram-positive strain, so that RNA could be selectively extracted from one strain [6, 9]. However, for most mixed species communities, selective extraction is not possible and a method needs to be developed in order to apply cDNA microarray technology.

Separating the target species from other community members before extracting RNA could be an approach in minimizing cross-hybridization on microarrays. Immuno-magnetic separation (IMS) using magnetic force to recover target cells with paramagnetic beads and specific antibodies has been widely used [13-15]. The IMS procedure has been standardized [16]. However, isolated cells had been used for different types of analysis such as microscopic imaging, PCR, and western blot, but never been considered for transcriptome analysis.

While the purity of recovered cells is important for microarray analysis, it was not always considered in previous studies. Preserving the transcription profile of target cells during IMS is critical for downstream microarray analysis. This is the most important concern addressed in this study. RNA*later* (Ambion, Austin, TX) has been used to stabilize and protect cellular RNA during sample storage. Combining the use of RNA*later* with IMS was desired to protect transcriptome during cell separation. However, the effect of RNA*later* on IMS separation efficiency had not been explored.

This study developed and tested a method that can be used to study the transcriptome of one species in a mixed species community, especially in biofilms. The efficiency of IMS

to separate a model species, *Escherichia coli*, from various suspended and biofilm cultures consisting of *E. coli* and *Stenotrophomonas maltophilia* was firstly evaluated. Recovery and purity of separated *E. coli* cells were reported. Changes in the transcription profile of *E. coli* cells due to sample processing and cell separation were quantified by cDNA microarray analyses and reported to evaluate the effectiveness of the developed method in this study.

4.3 Results and Discussion

4.3.1 Recovery rate of *E. coli*

Recovery rate of *E. coli* by immuno-magnetic separation (IMS) from a series of suspended cultures was firstly determined. A general antibody of *E. coli* (polyclonal anti-*E. coli* antibody (ViroStat, Portland, ME)) was used in this study. Using this antibody, recovery rate of *E. coli* was 74.4-98.2% when separated from suspended cultures with a density up to 1.9×10^8 CFU/ml (Figure 4.1). However, the recovery rate dropped to 60% for samples with ten-fold higher cells (1.9×10^9 CFU/ml), which may exceed the capacity of separation columns used in IMS (Figure 4.1). Therefore, *E. coli* cell densities in samples were adjusted to less than 2×10^8 CFU/ml for subsequent IMS.

Determining the recovery rate of target species is important when IMS is used to separate target species for subsequent cDNA microarray analysis. High recovery rates yield sufficient cells for RNA extraction, especially for low-abundance target species or from limited sample amounts. High recovery rates of *E. coli* were achieved from samples with a wide range of cell densities (10^4-10^8 CFU/ml). The recovery rates in this study are generally higher than those reported previously (53-82%) [17-19].

4.3.2 Purity of *E. coli* separated from dual-species cultures

Suspended mixtures containing 0.7-70% *E. coli* cells (10^4-10^6 CFU/ml *E. coli* and 10^5-10^8 CFU/ml *S. maltophilia*) were used to evaluate IMS for separating and purifying *E. coli* cells from various communities. One-step IMS enriched *E. coli* cells to a purity of over 95% from mixtures with 38.3-71.3% *E. coli* cells (Figure 4.2A). But the purity of *E. coli* cells after one-step IMS was too low to be acceptable (32-53%) when separated from mixtures containing less *E. coli* cells (0.7-13.4%) (Figure 4.2A). Therefore, a second step

of IMS was performed and *E. coli* cells were successfully enriched to a high purity of 95.9% from mixtures containing as little as 1.1% *E. coli* cells (Figure 4.2A).

Previous studies did not report whether other species, such as *S. maltophilia*, would bind to the anti-*E. coli* antibody [18-20]. The high purity of *E. coli* obtained by one- or two-step IMS (>95%) (Figure 4.2A) suggested that cross-reactivity, if there was any, was not a concern. Low purity of *E. coli* (32-53%) obtained from mixtures with small percentages of *E. coli* (0.7-13.4%) was a result of a small fraction (1%) of *S. maltophilia* cells accumulated in the LS columns, in which magnetically labeled *E. coli* cells were held during washing. When *S. maltophilia* was dominant in samples (e.g., *S. maltophilia* >90% and *E. coli* <10%), the seemly low accumulation of *S. maltophilia* (1%) yielded an absolutely high number of *S. maltophilia* cells, resulting in low purity of *E. coli* after IMS. However, since the accumulated *S. maltophilia* cells were not actually bound to the anti-*E. coli* antibody, they were removed during the second step of IMS, resulting in highly purified *E. coli* cells (Figure 4.2A).

Real dual-species biofilms harvested from flow cell systems were used to investigate whether IMS could also separate *E. coli* from biofilms. Biofilm matrix was homogenized to disperse cell aggregates into a suspension of single cells before IMS. Two independent separations were performed for aliquots of dispersed biofilms. Two-step IMS was able to enrich *E. coli* to around 95% from biofilms containing only 8.1% *E. coli* (2.3×10^6 CFU/ml *E. coli* and 2.6×10^7 CFU/ml *S. maltophilia*) (Figure 4.2B). The results demonstrated the feasibility of using IMS to separate *E. coli* cells from biofilms.

It is important to obtain target cells in high purity when separated from mixed species communities for subsequent cDNA microarray analysis, in order to effectively limit cross hybridization. The results showed that high purity of *E. coli* cells could be obtained by IMS from different mixed species communities (suspensions or biofilms) with various amounts of *E. coli* cells (0.7-70%).

4.3.3 Preservation of RNA integrity during cell separation

Preserving RNA integrity during IMS is critical when collected cells are used for subsequent cDNA microarray analysis. RNA*later* (Ambion, Austin, TX) has been used

widely to preserve RNA in bacterial cells, but the impact of RNA*later* on IMS performance was unknown. The recovery rate of *E. coli* dropped to 1% if samples remained in RNA*later* during the whole IMS procedure. It may be the result of antibody denaturation by global protein denaturing reagents in RNA*later*. Alternative products, such as RNAprotect (Qiagen, Germantown, MD), contain similar denaturing reagents and are expected to show similarly reduced recovery.

In order to overcome this problem, RNA*later* was removed during some procedure of IMS. Samples were stored in RNA*later* at 4°C overnight to allow the reagent to penetrate into bacterial cells and to stabilize intracellular RNA. RNA*later* was then removed and bacterial cells resuspended in separation buffer just before incubation with antibody and microbeads. One-step IMS could enrich *E. coli* to a similar level as shown in Figure 4.2A and remove over 99% of *S. maltophilia* cells (data not shown). The results confirmed that the modified protocol did not affect the recovery and purity of *E. coli* by IMS.

Pre-stabilization in RNA*later*, quick sample processing (~30 min), low working temperature (4 °C), and maintaining an RNAase-free environment were combined to limit RNA degradation during IMS, since RNA*later* had to be removed during some procedure of IMS. The effectiveness of these strategies in preserving the integrity of RNA was confirmed by observing high quality RNA extracted from IMS "sorted" cells using agarose gel electrophoresis (data not shown).

4.3.4 Impact of cell separation on *E. coli* transcription profiles

In order to evaluate whether gene expression profiles were changed during sample process (biofilm dispersion) and IMS cell sorting, cDNA microarray analysis was used to compare gene expressions of *E. coli* cells without dispersion and IMS (unsorted cells) and with dispersion and IMS (sorted cells). To eliminate possible impact of any non-target RNA (from the small amount (5%) of *S. maltophilia* cells remained in enriched collections), pure cultures of *E. coli* rather than dual-species mixtures were used to study changes in transcription profile of *E. coli* due to cell separation. The same procedure used for dual-species biofilm treatment, including cell dispersion and IMS, was performed with pure cultures of *E. coli*.

Differentially expressed genes were identified based on fold-change and statistical significance compared to the control (Figure 4.3) [21]. Only 10 and 45 of the 4,289 ORFs exhibited differential expression in two independent microarray studies I and II, respectively (each microarray study was performed with two technical replicates of microarray slides and each microarray slide had three built-in replicates). A complete list of the differentially expressed genes is provided in Appendix 4-1. Only eight of these genes showed consistent changes in both of the independent microarray studies (Table 4.1), with three genes up-regulated and five genes down-regulated in sorted *E. coli* cells in comparison to unsorted *E. coli* cells. The fold-change of gene expression ranged from 2.7 to -4.6 (Table 4.1).

It was not surprising to find genes with changed expression after several treatment steps, i.e., cell homogenization/dispersion, re-suspension in buffer, and IMS cell sorting. However, the number of genes that were differentially expressed was very low (eight genes correspond to 0.2% of the 4,289 ORFs). Thus, it can be concluded that transcription profiles of enriched *E. coli* cells were well preserved during IMS.

4.4 Conclusions

Good recovery, high purity and preserved transcription profiles of separated *E. coli* cells indicate that the method developed in this study can be used to study transcription profiles of *E. coli* in a mixed community with *S. maltophilia*. Although *S. maltophilia* was used as the non-target species in this study, this method can be used to remove other non-target species. In addition, the method should not be limited to studies of *E. coli*, but also other species of interest for which specific antibodies are available. While it will be important to determine antibody dosage and homogenization intensity in separating other species of interest, the basics of the method presented here can be applied to other communities. The applicability of the method to study real mixed-species communities has been tested by our recent study in identifying genes of *E. coli* involved in interactions with *S. maltophilia* (manuscript in preparation). Gene identification of species interactions can lead to further our understanding of mechanisms of species interactions as shown by previous studies [9]. The method developed here thus has the potential to

contribute to studies in which understanding the mechanisms of species interactions is an important component.

4.5 Materials and Methods

4.5.1 Bacterial strains and suspended mixtures

Overnight cultures of *E. coli* K-12 PHL644/pMP4655 (carrying a *gfp* gene under the control of a constitutive promoter) and *S. maltophilia*/pBPF-mCherry were grown in Luria-Bertani (LB) broth supplemented with tetracycline (80 μg/ml) or gentamicin (20 μg/ml) at 34°C with continuous shaking (200 rpm). Cells were pelleted by centrifugation (3,300×g, 4°C, 3 min), re-suspended, and diluted in 1× phosphate buffered saline (PBS, pH 7.4) supplied with 0.5% bovine serum albumin (BSA) (Pierce, Rockford, IL). A series of artificial mixtures of *E. coli* and *S. maltophilia* were prepared by mixing the PBS re-suspended and diluted *E. coli* and *S. maltophilia* cells at different ratios.

Biofilms were cultivated on the inner surface of silicon tubing (Cole-Parmer, Vernon Hills, IL) in a flow cell system as described previously [22]. Briefly, a flow cell system was assembled, sterilized, and conditioned by running 0.1× LB broth (10-fold diluted LB broth, 1 ml/min) at room temperature (20-25 ºC). Operation was paused for one hour to allow inoculation with *S. maltophilia* and *E. coli* mixed at a ratio of 1:1. After three days of growth, biofilms were scraped into 1× PBS and pre-homogenized on ice using a homogenizer (OMNI TH, Marietta, GA) set at the lowest speed for 30 seconds. Biofilms were further dispersed into single cells using the same homogenizer set at the maximum speed for two minutes. Over 99% of bacterial cells in the biofilm matrix were dispersed into single cells. The dispersed biofilm cells were then diluted in 1× PBS (with 0.5% BSA) for IMS.

4.5.2 Immuno-magnetic separation

One ml of samples was incubated with 10 μl anti-*E. coli* antibody (ViroStat, Portland, ME) for 10 min with gentle shaking. Bacterial cells were pelleted by centrifugation (3,300×g, 4 °C, 3 min) and re-suspended in 100 μl separating buffer (1× PBS, 0.5% BSA, 2 mM EDTA, pH 7.4) (EDTA: ethylenediaminetetraacetic acid). 10 μl streptavidin

microbeads (Miltenyi Biotec, Auburn, CA) were added and incubated at 4 °C in the dark for 10 min. Separation of *E. coli* cells was performed in LS columns and a midi MACS® separator (Miltenyi Biotech, Auburn, CA) following the protocol provided by the manufacturer, except that one more washing step was added to remove more *S. maltophilia* cells. In a two-step IMS, enriched cells from the first step IMS were directly transferred into a new LS column for the second separation. Densities of *E. coli* and *S. maltophilia* cells in samples and IMS enriched collections were measured using a plate-counting method with selective agar. Cell densities were used to calculate recovery and purity of *E. coli* after IMS.

The protocol was amended with the use of RNA*later* when enriched cells were used for microarray study. Bacterial cells were re-suspended in RNA*later* rather than PBS after sample collection and kept at 4°C overnight, followed by homogenization. RNA*later* was removed and cells were re-suspended in separating buffer just before IMS. During column separation, the buffer was additionally supplied with 10% (v/v) RNA*later*. Enriched cells were immediately stored in RNA*later*. The whole procedure was performed at 4°C. All buffers, reagents, and pipette tips were nuclease-free and pre-cooled.

4.5.3 Microarray study

Pure *E. coli* cultures were used to evaluate the effect of separation on transcriptome by microarray analysis. Suspended *E. coli* cultures were harvested from an annular reactor (1320 LJ, BioSurface Technologies, Bozeman, MT), supplied with 0.1× LB broth (100 ml/h) for 7 days after inoculation. Aggregates were removed from the cultures by filtration (5.0 μm Millipore, Billerica, MA). Suspended *E. coli* cells were immediately re-suspended in RNA*later* and stored at 4°C overnight. One aliquot of RNA*later* stored *E. coli* cells served as the control (“unsorted” cells) and was kept in RNA*later* without further treatment. The other aliquot was treated to acquire “sorted” cells as described above in the amended protocol. Samples collected independently from a second annular reactor served as a biological replicate for the microarray study.

RNA*later* was removed by filtration with a membrane (0.22 μm, Millipore, Billerica, MA) from *E. coli* cells just before RNA extraction for both "unsorted" and "sorted" cell collections. RNA extraction was based on a hot SDS/phenol protocol for RNA extraction [23]. A step of bead-beating (BioSpec, Bartlesville, OK) for one minute was added to break cells, and all chemical extractions were performed in phase lock gels (5 Prime, Fisher Scientific, Pittsburgh, PA). DNA was removed from extracted RNA with Turbo DNase treatment (Ambion, Austin, TX) at 37 °C for 30 min followed by purification with an RNeasy Mini Kit (Qiagen, Germantown, MD). The quality of RNA was examined by gel electrophoresis using E-gel with SYBR Safer (Invitrogen, Carlsbad, CA). RNA with high quality was further re-precipitated, concentrated, and stored at -80°C.

RNA was reversely transcribed into cDNA using random hexamers ($pd(N)_6$) (GE Healthcare, Piscataway, NJ) and labeled with Amersham CyDye Post-Labeling Reactive Dye (Amersham Biosciences, Piscataway, NJ) following the protocol provided by the Amino Allyl cDNA Labeling Kit (Ambion, Austin, TX). Quantity and labeling efficiency of cDNA was measured using a NanoDrop Spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE).

Microarray slides for *E. coli* were purchased from the University of Alberta (Edmonton, AB, Canada). Each slide contained three replicates of 5,978 70-mer oligonucleotides representing three *E. coli* strains (4,289 of them were for *E. coli* K-12). Sample preparation and loading, slide prehybridization, hybridization and washing were performed according to Corning protocols (GAPS II coated slides, Corning Inc., Lowell, MA). An extended 4-h prehybridization using a higher BSA concentration (1 mg/ml) was found to perform best in reducing background noise. Hybridization was in a Corning Microarray Hybridization Chamber (Corning Inc.) at 42 °C water bath.

Microarray slides were scanned with a Virtek ChipReader (Virtek Vision, Waterloo, ON, Canada). Spots on scanned images were recognized and pixel intensity for each spot was quantified using the TIGR software Spotfinder (v3.1.1). Gene expression data was analyzed in the software Acuity 4.0 (Molecular Devices, Sunnyvale, CA). LOWESS normalization was performed for every microarray with three iterations using a

smoothing factor of 0.4. Hybridized spots with oligonucleotides for strain *E. coli* K-12 having a high QC (quality control) value (>0.1), good flag tags (A, B and C) in both Cy3/Cy5 channels were chosen for further analysis. One sample t-tests were performed across replicates. Step-down Bonferroni-Holm was used for the correction of multiple hypotheses testing. Genes with at least two-fold change in expression (p-value < 0.05) were considered to have changed expression during sample dispersion and IMS. Microarray data was deposited in NCBI Gene Expression Omnibus database (GSE22885).

4.6 Figures and Tables

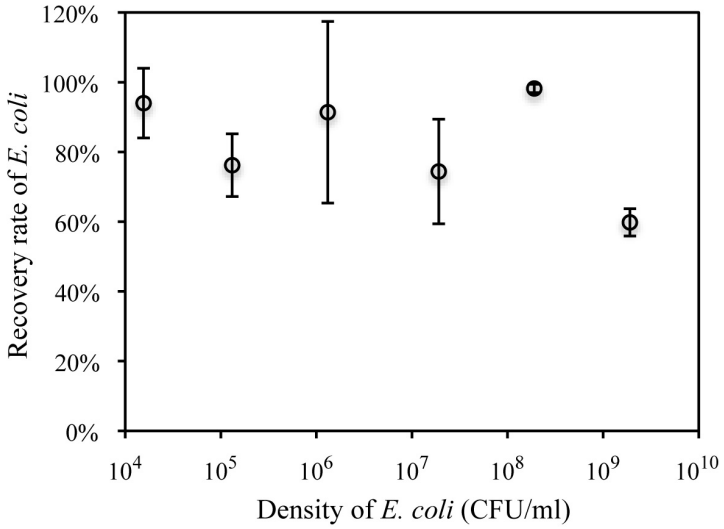

Figure 4.1 Recovery rates of *E. coli* cells after immuno-magnetic separation

Recovery rates of *E. coli* cells after one-step immuno-magnetic separation (IMS) from 1 ml suspensions of *E. coli* with densities adjusted from approximately 10^4 to 10^9 CFU/ml. Recovery rates were calculated as the percentage of recovered *E. coli* cells after IMS. Cell density was quantified with the method of plate count and volume of cell collections. Error bars indicate standard deviations of triplicate plate counts.

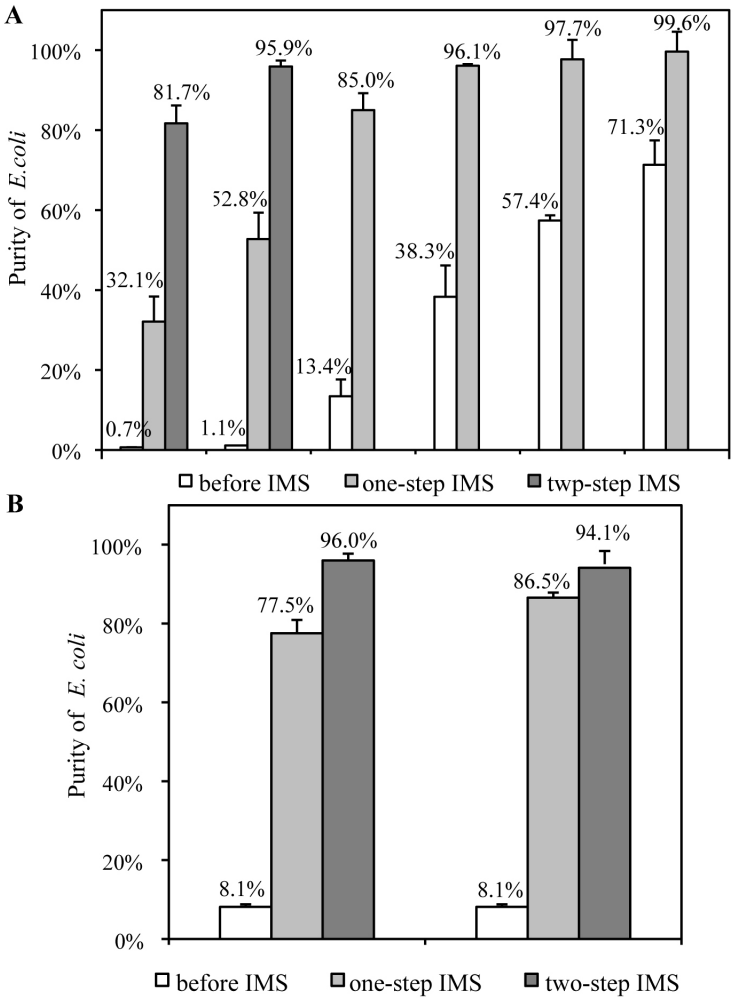

Figure 4.2 Purity of *E. coli* cells before and after immuno-magnetic separation from suspended mixtures and biofilms

Purity of *E. coli* cells before and after one- or two-step immuno-magnetic separation (IMS) from (A) suspended mixtures (B) biofilms of *E. coli* and *S. maltophilia* cells. Suspended mixtures were prepared by mixing suspended *E. coli* cells (10^4-10^6 CFU/ml) with *S. maltophilia* cells (10^5-10^8 CFU/ml) from planktonic cultures. Biofilms were scraped from a flow cell system and dispersed into suspensions of single cells (*E. coli* 2.3×10^6 CFU/ml, *S. maltophilia* 2.6×10^7 CFU/ml) before IMS. Cell densities of *E. coli* and *S. maltophilia* were quantified with the method of plate count with selective agar. Two independent IMS experiments were performed for aliquots of dispersed biofilms. Error bars indicate standard deviations of two or three replicate plate counts.

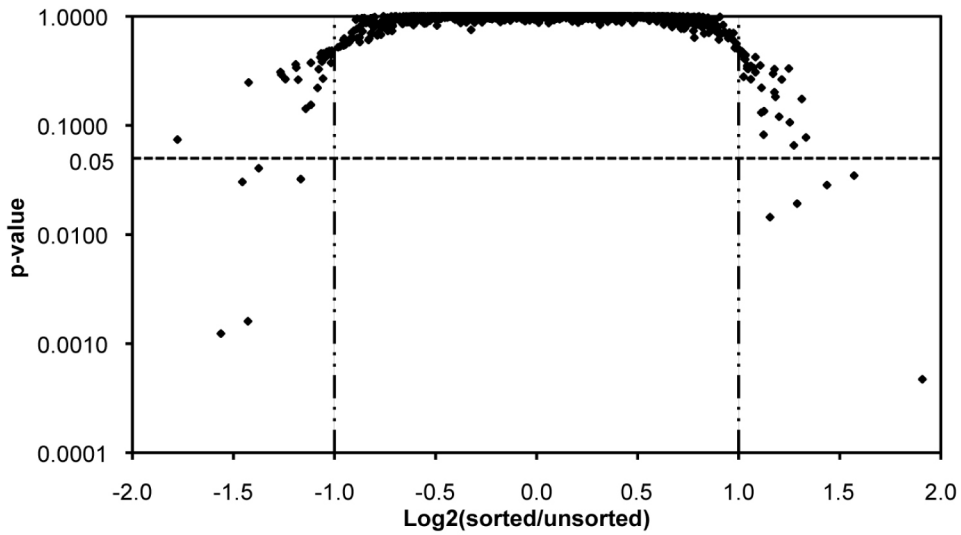

Figure 4.3 Plot of gene expression in sorted/unsorted cells

Plot of one-sample T-test p-values with fold-change in gene expression for all ORFs (open reading frames) of *E. coli* in microarray study I. A similar plot was acquired from another independent study II (not shown). Vertical lines show the cutoff of fold-change of 2 (Log_2 ratio of ±1), while the horizontal line shows the cutoff of p-value 0.05. Genes located in the left-bottom corner (Log_2 ratio <-1 and p-value <0.05) and in the right-bottom corner (Log_2 ratio >1 and p-value <0.05) were considered to have their expressions significantly changed due to the conduction of cell dispersion and IMS (immuno-magnetic separation) cell sorting. p-value was achieved by one sample student t-test for replicates of each gene. A total of ten genes were selected using these criteria, eight of which also differentially expressed in microarray study II.

Table 4.1 Genes differentially expressed in sorted *E. coli* cells

Genes with differential expressions[#] between IMS (immuno-magnetic separation) sorted *E. coli* K-12 cells versus unsorted *E. coli* K-12 cells[*]

Gene	Fold-change of expression (sorted/unsorted)		Description of gene product[⊕]
	Microarray study I	Microarray study II	
tldD	2.7 ± 1.4[Ψ]	2.7 ± 1.4	Predicted peptidase
proW	2.4 ± 1.1	3.3 ± 1.3	Glycine betaine transporter subunit
ansP	2.2 ± 1.1	2.5 ± 1.1	*L*-asparagine transporter
ydhB	-2.2 ± 1.1	-2.9 ± 1.2	Predicted DNA-binding transcriptional regulator
yhhN	-2.6 ± 1.3	-3.1 ± 1.2	Conserved inner membrane protein
ygeV	-2.7 ± 1.1	-3.3 ± 1.4	Predicted DNA-binding transcriptional regulator
flhE	-2.7 ± 1.2	-3.2 ± 1.2	Conserved protein
yicG	-3.0 ± 1.2	-4.6 ± 1.3	Conserved inner membrane protein

[#]Fold-changes of gene expression were significantly different from 2, with one-tail t-tests performed ($p < 0.05$).

[*]Sorted *E. coli* cells: *E. coli* cells treated with dispersion/homogenization and IMS cell sorting after pre-stored in RNA*later*; Unsorted *E. coli* cells: *E. coli* cells continuously stored in RNA*later* without any treatment.

[⊕]Description of gene product is updated according to records of *E. coli* K-12 MG1655 in NCBI Entrenz Gene Database.

[Ψ]Mean ± geometric standard deviation from two replicate slides, with three built-in replicates in each slide; positive and negative values indicate up- and down-regulation, respectively, in dispersed and IMS sorted cells. Geometric standard deviation=2^{SD}, where SD is standard deviation of $\log_2$ transformation of fold-change.

4.7 References

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Appendix 4-1 Full list of genes differentially expressed in sorted *E. coli* cells in two independent microarray studies

Full list of genes of *E. coli* K-12 differentially expressed in IMS (immuno-magnetic separation) sorted *E. coli* K-12 cells versus unsorted *E. coli* K-12 cells in two independent microarray studies I and II

Gene Name	Fold-change of gene expression (sorted/unsorted*)		Description of gene product$^{\oplus}$
	Microarray study I	Microarray study II	
tldD	2.7 ± 1.4$^{\Psi}$	2.7 ± 1.4	Predicted peptidase
proW	2.4 ± 1.1	3.3 ± 1.3	Glycine betaine transporter subunit
ansP	2.2 ± 1.1	2.5 ± 1.1	*L*-asparagine transporter
ydhB	-2.2 ± 1.1	-2.9 ± 1.2	Predicted DNA-binding transcriptional regulator
yhhN	-2.6 ± 1.3	-3.1 ± 1.2	Conserved inner membrane protein
ygeV	-2.7 ± 1.1	-3.3 ± 1.4	Predicted DNA-binding transcriptional regulator
flhE	-2.7 ± 1.2	-3.2 ± 1.2	Conserved protein
yicG	-3.0 ± 1.2	-4.6 ± 1.3	Conserved inner membrane protein
ybbO	3.0± 1.5	/	*L*-asparagine permease
aslA	3.8 ± 1.2	/	(p)ppGpp synthetase I/GTP pyrophosphokinase)
yihO	/	6.3 ± 1.4$^{\Psi}$	Predicted transporter
ydaJ	/	4.2 ± 1.4	Predicted peptidase
mhpT	/	4.0 ± 1.5	Predicted 3-hydroxyphenylpropionic transporter
metL	/	3.9 ± 1.5	Fused aspartokinase II
ldcA	/	3.6 ± 1.7	*L, D*-carboxypeptidase A
yciT	/	3.6 ± 1.3	Predicted DNA-binding transcriptional regulator
ytfB	/	3.4 ± 1.2	Predicted cell envelope opacity-associated protein
lpxD	/	3.4 ± 1.3	UDP-3-O-(3-hydroxymyristoyl)-glucosamine N-acyltransferase
rbsD	/	3.3 ± 1.2	Glycine betaine transporter subunit
arsC	/	3.1 ± 1.3	Predicted cytoplasmic sugar-binding protein
recC	/	3.1 ± 1.4	Exonuclease V

ydaK	/	3.0 ± 1.4	Putative transcriptional regulator
pabA	/	2.9 ± 1.5	Arsenate reductase
ilvM	/	2.9 ± 1.6	Predicted peptidase
gpmA	/	2.8 ± 1.4	Acetolactate synthase II, small subunit
relA	/	2.7 ± 1.5	Aminodeoxychorismate synthase, subunit II
yciQ	/	2.7 ± 1.2	Phosphoglyceromutase I
uxaC	/	2.7 ± 1.4	DNA-binding transcriptional regulator
hdfR	/	2.6 ± 1.3	Conserved protein
ynfH	/	2.5 ± 1.3	Uronate isomerase
flgF	/	2.4 ± 1.3	Acrylsulfatase-like enzyme
yfaE	/	-2.3 ± 1.2	Oxidoreductase, membrane subunit
ybhG	/	-2.3 ± 1.4	Flagellar basal body rod protein
actP	/	-2.4 ± 1.1	Predicted oxidoreductase
ybcN	/	-2.4 ± 1.1	Predicted protein
flgD	/	-2.6 ± 1.4	Conserved inner membrane protein
ybaW	/	-2.7 ± 1.3	Putative transporter subunit
pckA	/	-2.7 ± 1.2	Part of gsp divergon involved in type II protein secretion
fliM	/	-2.8 ± 1.5	Fused glutathionylspermidine synthetase/amidase
hyfA	/	-2.9 ± 1.2	Predicted fimbrial-like adhesin protein
narH	/	-2.9 ± 1.3	Conserved protein
yhbM	/	-3.0 ± 1.4	Predicted DNA-binding transcriptional regulator
yadL	/	-3.6 ± 1.4	Conserved inner membrane protein
gsp	/	-3.7 ± 1.1	Nitrate reductase I, beta subunit
gspB	/	-4.1 ± 1.4	Conserved protein
yehY	/	-4.2 ± 1.2	Predicted DNA-binding transcriptional regulator
ycgK	/	-6.4 ± 1.1	Flagellar motor switch protein

*Sorted *E. coli* K-12 cells: *E. coli* K-12 cells treated with dispersion/homogenization and IMS cell sorting after pre-stored in RNA*later*; Unsorted *E. coli* K-12 cells: *E. coli* K-12 cells continuously stored in RNA*later* without any treatment.

⊕Annotations are from NCBI Entrenz Gene Database, mostly limit to *E. coli* K-12 MG1655.

ΨMean ± geometric standard deviation from six replicates (two technical replicated slides × three built-in replicates per slide) for each gene; positive value indicates up-regulation in dispersed and IMS sorted cells, while negative value indicates down-regulation in dispersed and IMS sorted cells. Geometric standard deviation $=2^{SD}$, where SD is standard deviation of $\log_2$ transformation of fold-change.

/: not differentially expressed.

Chapter 5
Identifying Genes of *Escherichia coli* involved in Interactions with *Stenotrophomonas maltophilia* in Planktonic Cultures and Biofilms Using Transcriptome Analysis

5.1 Abstract

Biofilm formation by *Escherichia coli* was significantly inhibited when co-cultured with *Stenotrophomonas maltophilia* in static systems. cDNA microarray analysis was performed to identify genes of *E. coli* involved in species interactions with *S. maltophilia* in order to allow future studies of the mechanisms of altered *E. coli* biofilm formation. The transcription profile of *E. coli* in mixed cultures was compared to that in pure cultures by cDNA microarray technology after separating *E. coli* cells from mixed species cultures to eliminate cross hybridization of *S. maltophilia* transcript on *E. coli* microarray slides. A total of 89 and 108 genes were identified as differentially expressed in mixed species cultures when growing as biofilm and as planktonic cultures, respectively. Differential expression of certain identified genes was confirmed using *E. coli* reporter strains combined with single-cell based flow cytometry analysis. Co-culture with *S. maltophilia* affected genes involved in metabolism, signal transduction, cell wall composition, and biofilm formation of *E. coli.* The data suggest that these genes were likely involved in species interactions between *E. coli* and *S. maltophilia.*

5.2 Introduction

In this study, we used a transcriptomic approach to identify genes that were involved in species interactions affecting biofilm development. Microorganisms predominantly live as biofilms rather than in suspension in most environmental, industrial and medical systems [1]. These biofilms are rarely composed of single species, but rather consist of multiple co-existing species with various intra- and inter-species interactions [2]. Observations of species interactions and their effects on biofilm formation have been widely reported [3-5]. Species exhibit interactions as commensal relationships [6], co-

aggregation [7], and can also communicate through quorum sensing signals [8], inhibitory compounds [9], and surface associated proteins [10].

Escherichia coli and *Stenotrophomonas maltophilia* are frequently detected in water, soil, sludge as well as urinary tract infections [11-13]. Co-isolation of the two species suggests that they may occupy similar ecological niches [14]. Our previous study (Chapter 3) found that *E. coli* biofilm formation was inhibited in co-culture with *S. maltophilia* in static batch culture, and the inhibition was not mainly due to diffusible signals. This study evaluated the effects of co-culture on *E. coli* cells at the gene expression level.

Identification of genes involved in species interactions could promote the mechanistic study of species interactions. cDNA microarray technology is a high-throughput method for relative gene expression analysis and identification of important genes. It has been applied to study dual-species communities [15, 16]. These transcriptome studies have lead to a better understanding of the mechanisms of species interactions, such as stabilized arginine synthesis in *Streptococcus gordonii* by *Actinomyces naeslundii* [17] or supply of iron for *Pseudomonas aeruginosa* by *Staphylococcus aureus* [15]. However, variable cross-hybridization of non-target species with the microarray for species of interest was observed in microarray studies of mixed species cultures [15, 16]. This problem was addressed by developing a method to separate target species from mixed species communities, while preserving the transcriptome of the target species (Chapter 4). This study applied this separation method and cDNA microarray technology to identify genes of *E. coli* potentially involved in species interactions with *S. maltophilia* in both planktonic cultures and biofilms.

5.3 Results

The effects of *S. maltophilia* on the growth of *E. coli* in planktonic and biofilm cultures were studied in Chapter 3. *E. coli* formed thick biofilms on inert surface in pure cultures (Figure 3.2). However, in co-cultures, *E. coli* only formed small microcolonies interspersed on the surface covered by a single layer of *S. maltophilia* cells (Figure 3.2D). The biomass of *E. coli* in mixed species biofilms was 70-90% lower than that in single species biofilm (Figure 3.2A). Previous study in Chapter 3 revealed that the inhibited *E.*

coli biofilm formation resulted from nutrient competition, reduced cell aggregation, and outcompeted surface attachment by *S. maltophilia.* It also indicated that species interactions and the inhibitive effect on *E. coli* biofilm formation required cell-to-cell contact between them.

This study intended to understand the effect of species interactions on *E. coli* cells from the transcription level. Changes in the *E. coli* transcriptome may uncover genetic mechanisms of inhibited *E. coli* biofilm formation in mixed species culture with *S. maltophilia*.

5.3.1 *E. coli* genes differentially expressed in co-cultures

cDNA microarray technology was used to identify *E. coli* genes differentially expressed in co-cultures, assuming that these differentially expressed genes may be involved in species interactions. Prior to cDNA microarray analysis, *E. coli* cells were separated from mixed species cultures before RNA extraction to reduce cross-hybridization using a method as previously described (Chapter 4). Two sets of microarrays were performed for different growth modes, biofilms and planktonic cultures. Gene expression profile of *E. coli* in mixed species biofilms was compared to that of *E. coli* in pure cultured biofilms ("Biofilm Mixed vs Pure"). A total of 89 genes (83 up- and 6 down-regulated) were identified as differentially expressed in biofilms due to co-cultures with *S. maltophilia* (Figure 5.1, Table 5.2 and Appendix 5-1). The second set of microarrays examined growth mode of planktonic cultures ("Planktonic Mixed vs Pure") and identified 108 differentially expressed genes due to co-cultures (104 up-and 4 down-regulated) (Figure 5.1, Table 5.2 and Appendix 5-2). Comparison of the two lists of identified genes showed 65 genes in common, which may be involved in species interactions regardless of growth mode. And the other 24 and 43 genes were likely to be specific to species interactions in biofilms and planktonic cultures, respectively.

An additional set of microarrays was performed to identify genes differentially expressed due to different growth modes in pure cultures ("Pure Biofilm vs Planktonic"). Of the 138 genes identified, only eleven were previously screened as candidate genes of species interactions from the first two sets of microarrays (Figure 5.1). Expression of the eleven

genes is shown in Table 5.3. Most of them (10 out of 11) exhibited opposite regulation trends in the two sets of microarrays (Biofilm Mixed vs Pure, and Pure Biofilm vs Planktonic).

Candidate genes for species interactions were categorized based on protein functions according to the clusters of orthologous groups tag (COG tag) (Figure 5.2). More than 40% of genes were grouped in the category of metabolism, including but not limited to transport and metabolism of carbohydrate, inorganic ion, amino acid, and secondary metabolites, in both growth modes. Around 15% and 18% were categorized as cellular process/signaling (e.g. cell wall and membrane, cell motility) and information storage/processing (e.g. replication and transcription) genes, respectively. The left 25% of identified genes were poorly or un-categorized.

5.3.2 Confirmation of differential gene expression

Differential expression of identified genes in mixed species cultures (Table 5.2 and Appendix 5-1,5-2) was confirmed using *E. coli* transcriptional reporter strains. Limited by the availability of all corresponding reporter strains and those strains that exhibited green fluorescence intensity higher than the background of *S. maltophilia*, a total of seventeen and fourteen genes were finally available for gene expression confirmation test in planktonic cultures and biofilms, respectively. Eight reporter strains showed increased fluorescence in planktonic co-cultures compared to pure cultures, confirming the up-regulation of these genes in mixed species planktonic culture (Figure 5.3A). While in biofilms, eight genes were confirmed as up-regulated and one as down-regulated when *E. coli* cells were co-cultured with *S. maltophilia* (Figure 5.2B).

5.3.3 Verification of genes involved in species interactions

It was hypothesized that some, if not all, differentially expressed genes in co-cultured *E. coli* cells were involved in real interactions with *S. maltophilia.* To test this hypothesis, we examined biofilm formation by *E. coli* knockout mutants of fifteen genes in pure and mixed cultures. The fifteen genes were selected based on their highly changed expression and the availability of corresponding knockout mutants in the Keio library [18].

Each knockout mutant was compared with wild type *E. coli* strain in biofilm formation in mixed and single species cultures. Mixed species biofilms of *S. maltophilia* with five knockout mutant strains (Δ*yddB*, Δ*fliA*, Δ*phoH*, Δ*sapA*, and Δ*cspA*) showed significantly higher biomass as compared to wild type *E. coli*-*S. maltophilia* biofilm (Figure 5.4A). Microscopic inspection found that increased overall biomass of mixed species biofilms resulted from increased *E. coli* knockout mutants (data not shown). The other ten knockout mutant strains showed no difference from wild type. Interestingly, the five mutants (Δ*yddB*, Δ*fliA*, Δ*phoH*, Δ*sapA*, and Δ*cspA*) showed no significant difference from wild type *E. coli* in pure biofilm formation (Figure 5.4B). The results further confirmed that these genes were involved in species interactions, which inhibited biofilm formation of *E. coli* in co-cultures.

5.4 Discussion

We observed a significantly inhibited biofilm formation of *E. coli* PHL644 when co-cultured with *S. maltophilia* in microtiter plates (Figure 3.2). Further study showed that *E. coli* biofilm inhibition was not due to diffusible signals produced by *S. maltophilia,* although the signals was shown to regulate biofilm formation of other species in other studies [19]. This highlights the importance of direct cell-contact based interactions between *E. coli* and *S. maltophilia* in the *E. coli* biofilm formation. To further study the mechanisms of species interactions, genes that are potentially involved in species interactions were identified through a transcriptomic approach.

5.4.1 Transcriptome analysis and confirmation of differential gene expression

Transcriptome analysis in this study was focused on *E. coli* since biofilm formation of *E. coli* in co-cultures was affected while no effects on *S. maltophilia* biofilm formation were observed (Figure 3.2). Transcriptome analysis of *E. coli* in co-cultures was achieved using a previously developed sorting method (Chapter 4), prior to cDNA microarray analysis, to eliminate cross-hybridization from *S. maltophilia*. Two sets of DNA microarrays were performed to identify differentially expressed genes of *E. coli* in co-cultures in two growth modes: biofilms and planktonic cultures. Most indentified genes were up-regulated (83 out of 89 in biofilms, 104 out of 108 genes in planktonic cultures)

(Figure 5.1). This result was not due to bias in dye labeling since dyes were swapped to label cDNA in the two sets of microarrays. It was neither an arbitrary effect in data analysis since normalization was performed. Moreover, the third set of microarrays identified more down-regulated genes using the same method of data analysis (37 down-regulated genes in "Pure biofilm vs planktonic") (Figure 5.1).

E. coli transcription reporter strains combined with flow cytometry analysis were used to confirm differential expression of identified genes in mixed species and pure cultures. Single-cell based flow cytometry analysis was able to selectively quantify fluorescence of *E. coli* cells without the need to separate them for mixed species cultures, as long as *E. coli* reporter strains showed higher fluorescence than that of *S. maltophilia* cells in this study. However, this confirmation approach was limited by the fact that not all identified genes had a corresponding reporter strain and not all available *E. coli* reporter strains showed higher fluorescence than the background fluorescence of *S. maltophilia*. Therefore, only a subset of genes (17 for planktonic and 14 for biofilm culture) was selected for confirmation in this study. Of the selected genes, 50-60% were confirmed as up- or down-regulated, which was in agreement with transcriptome analysis. Confirmation of differential expression of these identified genes in both biofilm and planktonic cultures validated the feasibility of cDNA microarray analysis for identifying genes that were involved in species interactions in mixed species cultures.

Based on genes identified by cDNA microarray analysis, in the following section, we are able to deduce *E. coli* response when co-cultured with *S. maltophilia*.

5.4.2 The effect of co-culture on *E. coli* metabolism

Metabolism plays an important role in species interactions. Substrate/nutrient competition and utilization between various species in co-cultures has been previously studied [20]. Specifically in commensal relationships, secondary metabolites from one species may serve as important carbon source for another species [6, 21, 22]. Thus it was not surprising that in this study, a large amount of genes differentially expressed in co-cultures (>40%) were related to metabolism (Figure 5.2). Most of genes were differentially expressed in both growth modes (Table 5.2 and Appendix 5-1,5-2).

Metabolism of *E. coli* cells was affected at the level of gene expression, although planktonic growth (cell density) of *E. coli* in mixed species cultures was not significantly reduced (data not shown).

Several genes involved in carbohydrate metabolism were differentially expressed. Specific carbohydrate transport genes (*araH, dgoT, rhmT,* and *ytfR*) and genes of the phosphotransferase system (*nagE, yadI, gnd,* and *chbB*) were induced when *E. coli* was co-cultured with *S. maltophilia* (Table 5.2). Induction of genes involved in an alternative sugar uptake system was also reported in previous studies of mixed species cultures [16]. In this study, up-regulation of these metabolism genes may result from carbohydrate competition of *E. coli* with *S. maltophilia* or the utilization of secondary metabolites produced by *S. maltophilia* due to primary carbon starvation at the time of cell harvesting (early stationary phase). It was unknown how differential expression of these genes may directly affect biofilm formation of *E. coli*, but various carbohydrate metabolism genes were related with biofilm formation of several species in previous studies [23, 24].

Inorganic ion transport genes involved in iron (*ftnA*, *fecR*), sulfur (*ydeN*), and phosphate (*phnL*, *pitA*) as well as some predicted ion transporters (*yddB*, *yjcE*, *yfbS*) were induced in mixed species cultures. Corresponding to these transporters, phosphate metabolism gene *phoH* and sulfur metabolism genes *sirA* and *cysD* were also induced in co-cultures. Ferritin A (FtnA) is an iron storage protein scavenging iron from disrupted iron-sulfur clusters. Stored iron can then be used in the presence of thioredoxin reductase system [25]. Gene *ftnA, fecR* and thioredoxin gene *trxA* were all up-regulated. Iron was not supplemented specifically but may exist as trace element in the complex Luria Broth media. Up-regulation of iron scavenging, transport and utilization genes suggested that *E. coli* may compete with *S. maltophilia* for iron in co-cultures. Expression of gene *phoH* is induced in the starvation of phosphate. In this study up-regulation of *phoH* was observed in mixed species biofilms, suggesting that *E. coli* may encounter phosphate depletion in biofilms when co-cultured with *S. maltophilia.* Induction of iron, phosphate and other ions was also observed in other species of interest rather than *E. coli* in previous studies of mixed species cultures [15, 16]. While most mechanisms underlying the observation

were not fully explored, iron provision by one species to the other in co-cultures was a well-studied example of species interactions [15].

5.4.3 Effect on signal transduction and cell wall component of *E. coli*

S. maltophilia produces diffusible signal factors (DSF), which are a group of fatty acids [26]. DSF could serve the purpose of inter-species signaling and affect biofilm formation of other species such as *Pseudomonas aeruginosa* [19]. However in this study, DSF, if produced, in supernatant of *S. maltophilia* or cell contact-free cultures failed to show any significant effect on biofilm formation of *E. coli*. This study found that a fatty-acid transporter gene, *fadD*, of *E. coli* was up-regulated in both growth modes of co-cultures. Gene *fadD* encodes a fatty acyl-CoA synthetase and transports fatty acid across the inner membrane. Acyl-CoA is a key intermediate of fatty acid metabolism. Induction of the key gene *fadD* suggests that *E. coli* may digest fatty acid signals produced by *S. maltophilia.*

Another group of identified genes with differential expression in co-cultures was for cellular process and signaling of *E. coli* (Figure 5.2). These genes indicated that *E. coli* sensed and responded to changed environments due to co-culture with *S. maltophilia*. For example, two-component sensor genes *envZ* and *kdpD* were up-regulated in co-cultures. EnvZ is a membrane-bound sensor kinase. With the response regulator OmpR, it could sense and react to environmental changes such as osmotic variations [27]. Induced expression of *envZ* indicated that *E. coli* sensed changed environment in co-culture with *S. maltophilia*, which may affect biofilm formation of *E. coli* since the response gene *ompR* was shown to influence curli production and biofilm formation of *E. coli* PHL644 in static cultures [28].

Peptidoglycan is an important component of bacterial cell wall. Several *E. coli* genes related to peptidoglycan biosynthesis such as *ampH, ddpX, ddpA, ycfS* and *dacA* were up-regulated in co-cultures. The induction of *ampH* was as high as 23-fold. Gene *ampH* encodes an uncharacterized protein related to class C β-lactamases with penicillin binding ability. Deletion of this gene affected normal morphology of *E. coli* cells [29]. Product of gene *ddpX* is a dipeptidase that catalyzes the hydrolysis of D-alanyl-D-alaline

dipeptide for biosynthesis of peptidoglycan, while *ddpA* is a D-alanine-D-alanine transporter. YcfS is responsible for the attachment of Braun lipoprotein to peptidoglycan and contributes to the integrity of outer envelope structure of *E. coli* [30]. Gene *dacA* encodes a carboxypeptidase and contributes to the maintenance of normal morphology of *E. coli.* Over-expression of *dacA* converts *E. coli* from rod-shape to spherical form [31]. It was unknown what triggered the induction of these peptidoglycan genes of *E. coli* in co-cultures. A previous study observed cell lysis of *Lactococcus lactis* in mixed species cultures and found that several genes encoding peptidoglycan hydrolases were induced while none of the biosynthesis genes were induced [16]. In contrast, this study found that several peptidoglycan biosynthesis genes were up-regulated, suggesting possible anti-lysis of *E. coli* cells in co-cultures by induction of peptidoglycan biosynthesis.

5.4.4 Reduced biofilm formation of *E. coli* in mixed species culture

An observable effect of species interactions between *E. coli* and *S. maltophilia* was the significantly reduced biofilm formation of *E. coli* in co-cultures. One approach to identify genes responsible for the phenomenon was to find common genes differentially expressed both due to species interactions and due to different growth modes. Eleven genes were screened using this approach and ten of them showed opposite expression trends in the two sets of microarrays (Table 5.3). Nine genes (*phoH, bolA, narZ, hspQ, gadA, yeaH, rpiR, ygiM* and *gadC)* were down-regulated in pure biofilms compared to pure planktonic cultures. It indicated that *E. coli* cells with low expression of these genes tend to form biofilms and cells with high expression tend to switch to planktonic growth. Expression of these genes all showed higher levels in mixed species biofilms than in pure biofilms, possibly due to species interactions. Up-regulation of these genes may trigger *E. coli* cells to switch from attached growth to planktonic growth, probably resulting in biofilm detach and reduction in biomass as we observed in this study. This assumption was partially confirmed for the gene *bolA* using an *E. coli* strain with overexpressed *bolA* (data not shown).

Most identified *E. coli* genes involved in species interactions were up-regulated in co-cultures while biofilm formation of *E. coli* was significantly reduced in co-cultures (Figure 5.1). This result suggested that knockout of these genes might reduce the

response of *E. coli* to the presence of *S. maltophilia* and would restore at least partially the phenotype of robust biofilm formation of *E. coli* PHL644 as observed in pure cultures (Figure 5.4). The hypothesis was verified by the observation of increased biofilm formation by five *E. coli* knockout mutant strains (Δ*yddB,* Δ*fliA,* Δ*phoH,* Δ*sapA* and Δ*cspA*) compared to the wild type strain in the presence of *S. maltophilia* (Figure 5.4). This confirmed that these genes were involved in species interactions and their up-regulation resulted reduced biofilm formation of *E. coli,* since knocking out them broke partial of species interactions and the effect of initiative biofilm formation of *E. coli.* How each of these five genes was involved in interactions with *S. maltophilia* and why their knocking out resulted in increased *E. coli* biofilm formation required detailed study in future.

5.5 Conclusions

This study applied cDNA microarray technology for transcriptome analysis of *E. coli* in mixed-species communities and identified genes that were potentially involved in species interactions with *S. maltophilia*. These genes showed genetic responses of *E. coli* to *S. maltophilia* co-culture in metabolism, signal transduction, and cell wall component etc. Differential expression of some identified genes in mixed species culture was confirmed using *E. coli* transcription reporter strains by flow cytometry. Some genes were shown to affect biofilm formation of *E. coli* in mixed species cultures. To the best of our knowledge, this is the first study to analyze the transcription profile of a Gram-negative species co-cultured with another Gram-negative species, since cross-hybridization is an issue for transcriptome analysis of mixed species community with both gram-negative species for which selective RNA extract is impossible. The approach used in this study should be very useful to study species interactions in other mixed species communities in many engineered and natural environments. Identification of genes that are potentially involved in species interactions will lead to further study of genetic mechanisms of species interactions. Characterization of identified genes will provide insights into genetic mechanisms of the response of *E. coli* to the presence of *S. maltophilia* in mixed species planktonic cultures and biofilms. In future, it would be interesting to study how *S.*

maltophilia responded to the presence of *E. coli* in mixed cultures as well, which could provide more insights into the interactions between *S. maltophilia* and *E. coli*.

5.6 Materials and Methods

5.6.1 Bacterial strains and cultures

All strains and plasmids used in this study are listed in Table 5.1. Following antibiotics were used when appropriate (Sigma-Aldrich, St. Louis, MO): tetracycline (20 μg/ml), gentamicin (20 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (30 μg/ml). Planktonic cultures were grown in 10-fold diluted Luria Broth (0.1×LB) with continuous shaking at room temperature (20 °C) for 18 h.

Biofilms were grown in 96-well Nunclon plates (Fisher Scientific, Pittsburgh, PA) for biomass quantification and in glass-bottom plates (MatTek, Ashland, MA) for microscopic imaging. Petri dishes and 6-well plates with large surface area were used to harvest biofilm biomass. Biofilms were cultured under static conditions for 18 h at room temperature for gene-expression analysis or 22-24 h for biofilm imaging analysis. Inoculated *E. coli* to *S. maltophilia* ratio was approximately 1:1 except for microarray studies, in which the ratio was adjusted (5:1 ~ 2:1) to obtain about 50% of *E. coli* cells in mixed species cultures.

Contact-free cultures were performed in Transwells (Corning, Lowell, MA) with a polycarbonate membrane (0.4 μm) to separate cells while still allowing *S. maltophilia* and *E. coli* interactions through diffusible signals. *S. maltophilia* was cultured in the insert while *E. coli* in the 24-well plate.

5.6.2 Quantification and imaging of biofilms

Biomass of biofilms was quantified using the microtiter assay with crystal violet staining as described previously [32]. Biofilms were examined with a confocal laser scanning microscopy (CLSM) (Olympus, Center Valley, PA). *E. coli* labeled with green fluorescence protein (GFP) and *S. maltophilia* labeled with red fluorescent protein mCherry were imaged in green and red channel with appropriate filter sets, respectively.

For imaging of biofilms developed by non-labeled bacterial strains, biofilms were stained with 5 μM SYTO 9 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

5.6.3 Microarray analysis

Samples were immediately re-suspended in RNA*later* (Applied Biosystems, Austin, TX) after harvest and stored at 4 °C overnight. *E. coli* cells were separated from samples using a previously developed method (Chapter 4). Briefly, cells were dispersed with a homogenizer, re-suspended in buffer for incubation with anti-*E. coli* antibody (ViroStat, Portland, ME) and microbeads, and finally separated using a midi MACS® separator (Miltenyi Biotec, Auburn, CA). Enriched cells were re-suspended in RNA*later* until RNA extraction. Pure *E. coli* cultures were processed with the same protocol to normalize the effect of separation on transcription profiles.

Enriched cells were recovered from RNA*later* just before RNA extraction with a hot SDS/phenol protocol modified by adding a 1-min bead-beating (http://www.genome.wisc.edu/pub/reprints/GEC_RNA_purification_ecoli.pdf).
Extractions were digested with DNase and purified with an RNeasy Mini Kit (Qiagen, Germantown, MD). Indirect labeling was performed using an Amino Allyl cDNA Labeling Kit (Ambion, Austin, TX) according to the manufacturer's protocol with random hexamers ($pd(N)_6$) (GE Healthcare, Piscataway, NJ).

E. coli microarray slides were purchased from the University of Alberta (Edmonton, AB, Canada). Each slide contained 4,289 oligonucleotides for *E. coli* K-12. Sample preparation and slide hybridization were performed according to the manufacturers protocol (Corning, Lowell, MA). Three sets of microarray experiments were performed, each with two biological, two technical, and three slide built-in replicates, resulting in twelve replicates for each gene.

Slides were scanned and images were analyzed using Spotfinder (TIGR). Data analysis was performed using Acuity 4.0 (Molecular Devices, Sunnyvale, CA). Spots for strain *E. coli* K-12 with quality control value larger than 0.1 and good flag tags in both channels were selected for analysis. LOWESS normalization was performed for every microarray

with three iterations using a smoothing factor of 0.4. Genes with at least two-fold change in expression (p-value < 0.05) were considered as differentially expressed. Gene annotation and COG tag was updated according to NCBI (http://www.ncbi.nlm.nih.gov/). Microarray data will be deposited into NCBI Gene Expression Omnibus database.

5.6.4 Confirmation of gene expression

Expression of identified genes was confirmed using *E. coli* reporter strains in a promoter library (PEC3877, Pittsburgh, PA), each with a *gfp* fused to the promoter of an *E. coli* gene. Flow cytometry (Beckman Coulter, Brea, CA) was used to compare GFP fluorescence of a reporter strain in pure- with mixed-species cultures. *S. maltophilia* cells were set as background. Cells with higher fluorescence than the background were considered as *E. coli* and were collected to calculate mean fluorescence. At least two replicates were performed for each reporter strain.

5.7 Figures and Tables

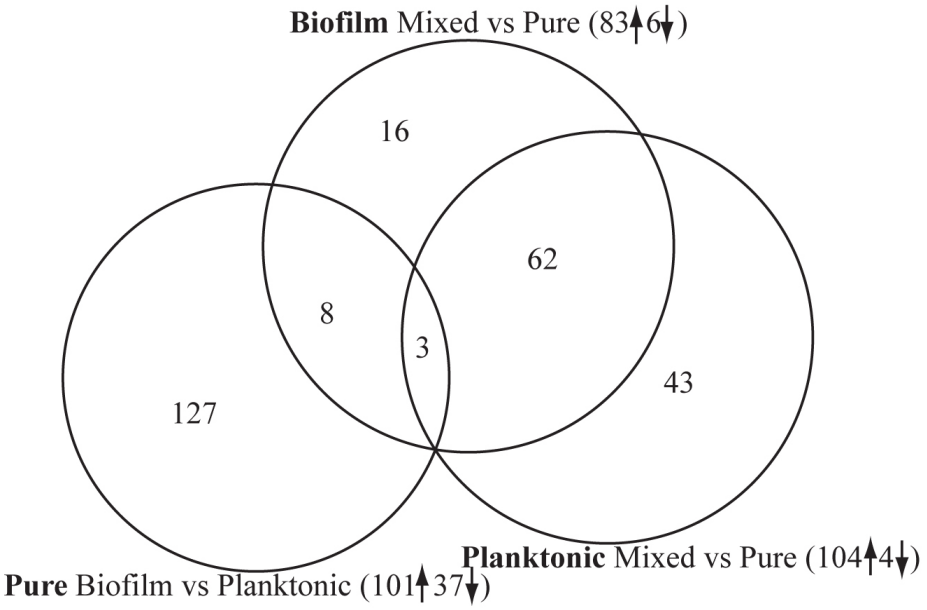

Figure 5.1 Venn diagram of the number of *E. coli* genes identified in microarrays

Each circle in the Venn diagram represents one set of microarray experiment. *Biofilm (or Planktonic) Mixed vs Pure* is the set comparing *E. coli* transcription profile in mixed species biofilms (or planktonic cultures) with that in pure *E. coli* biofilms (or planktonic cultures). *Pure Biofilm vs Planktonic* is the set comparing biofilms with planktonic cultures of pure *E. coli*. The number of identified genes was indicated in the parentheses, with up and down arrows showing up- and down-regulation of genes. Numbers in the circles show genes identified in either one or multiple sets of microarrays. Genes with at least 2-fold change in expression at a significant level of p-value<0.05 were considered as differentially expressed and identified in microarrays.

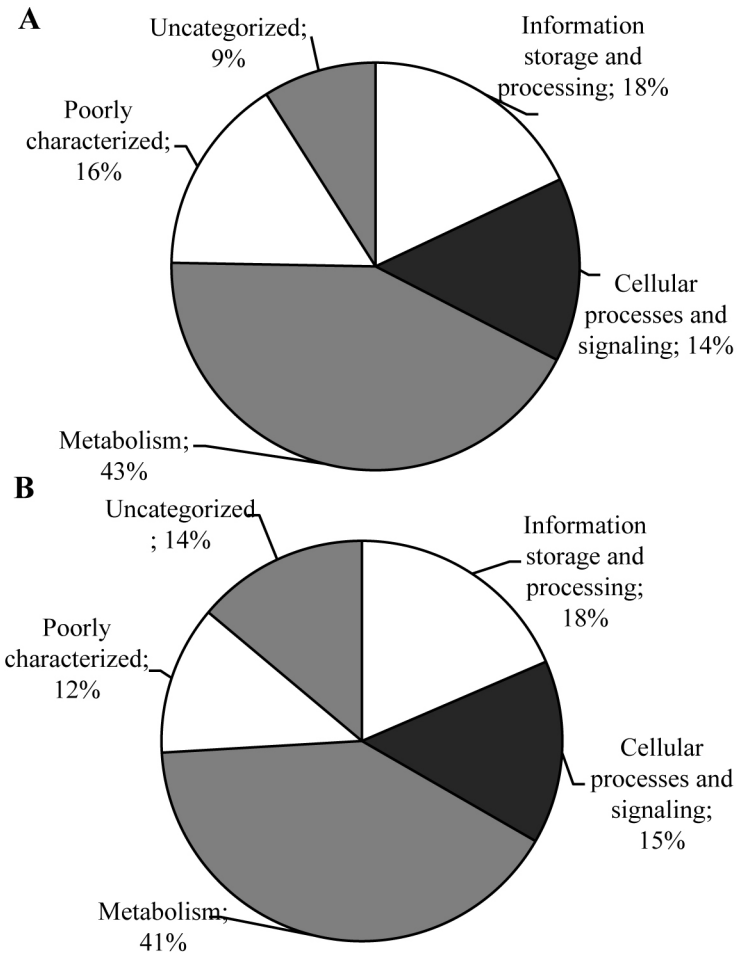

Figure 5.2 Categorization of identified genes involved in species interactions

Categories of identified genes identified by microarray analysis and potentially involved in species interactions in (A) biofilms and (B) in planktonic cultures. Genes were classified based on their protein functions according to their COG tag (clusters of orthologous groups).

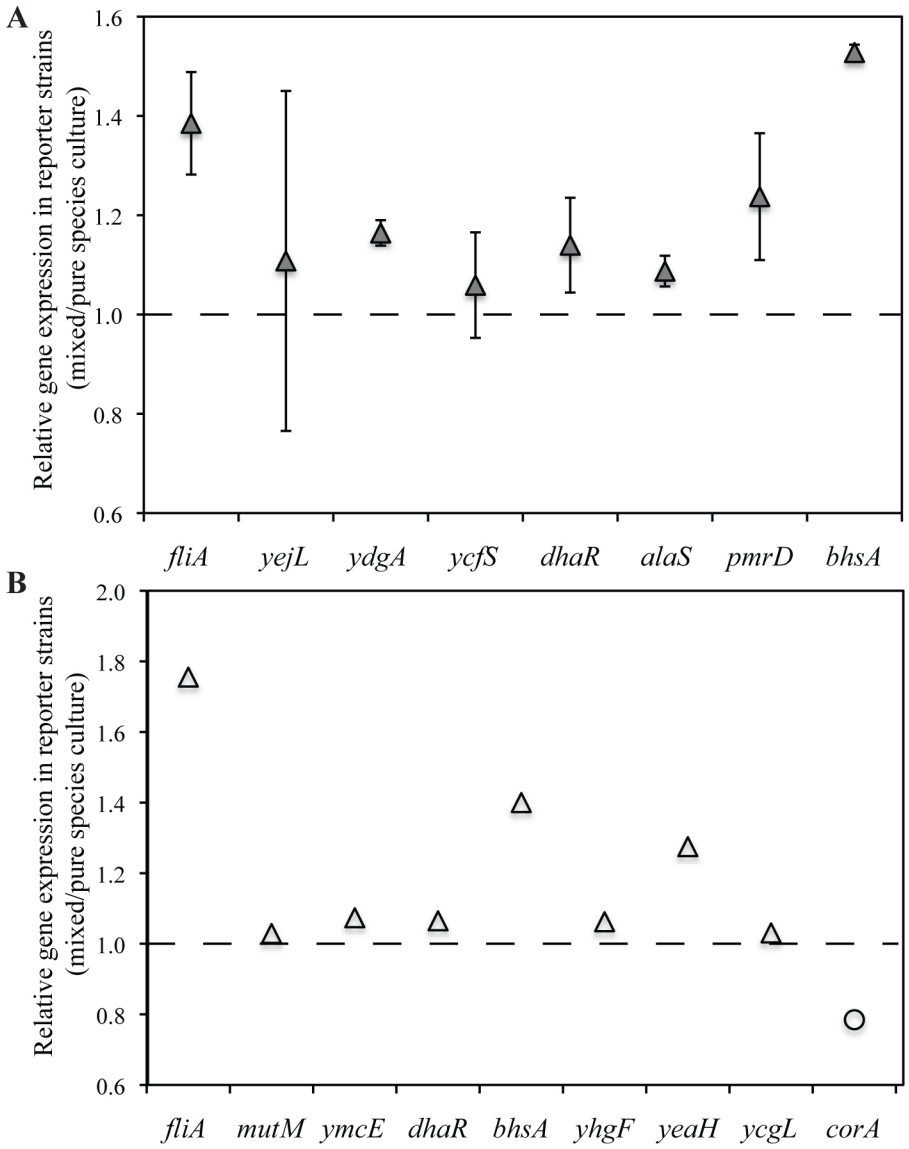

Figure 5.3 Confirmation of differential expression of some genes of *E. coli* identified by microarray analysis

Confirmed differential expression of *E. coli* genes in (A) mixed species planktonic cultures and (B) in mixed species biofilms, as normalized to corresponding pure *E. coli* planktonic and biofilm cultures, respectively. Each reporter strain carried a plasmid with a green fluorescence protein (*gfp*) gene under the control of the promoter of a gene. Gene expression was thus measured as the fluorescence of reporter strains in mixed or single species cultures by flow cytometry. Symbols triangle and circle mean up- and down-regulation of that gene according to microarray analysis, respectively. Error bars are standard deviations of two to four independent replicates.

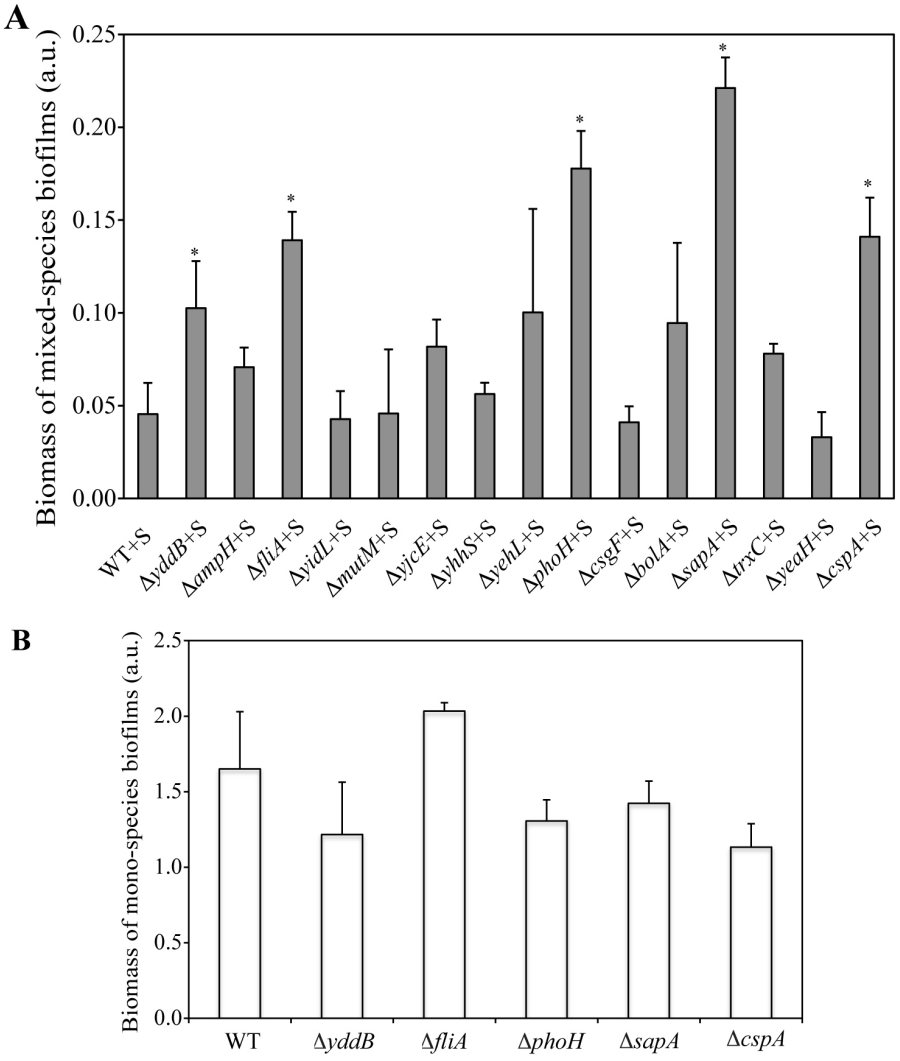

Figure 5.4 Effect of gene deletion on biofilm formation of *E. coli* cells in mixed and mono-species cultures with *S. maltophilia*

Biomass of (A) mixed species biofilms of wild type *E. coli* (WT) or single gene knockout mutant (Δ*yddB* etc.) with *S. maltophilia* (dedicated as S in the figure) and (B) mono-species biofilm of wild type *E. coli* or knockout mutants. Biofilms were cultured in microtiter plates and biomass was quantified by staining with crystal violet and expressed in arbitrary unit (a.u.). Error bars were standard deviation of at least three replicates. Asterisk * indicated significant difference ($p<0.05$) comparing to "WT+S".

Table 5.1 Plasmids and strains used in this study

Strains or plasmids	Genotype	Source or reference
pBPF-mCherry	mCherry fused to the *oprF* promoter in the vector pBBR1MCS-5	LIamas et al. [33]
pMP4655	*egfp* fused to the *lac* promoter in the vector pME6010	Bloemberg et al. [34]
E. coli K-12 PHL644	MC4100 *malA-kan ompR234,* increased curli expression	Vidal et al. [28]
S. maltophilia	Environmental isolate	This study
E. coli K-12 BW25113		CGSC # 7636
E. coli reporter strains	*E. coli* K-12 MG1655 containing the reporter plasmid pUA66 or pUA139 carrying a *gfp*-fusion with the promoter of each corresponding genes including: *acpP, acpS, alas, bhsA, bolA, corA, crl, cspA, dhaR, fliA, folA, gnd, mutM, phoH, pitA, pmrD, pntA, trxA, ycfA, ycgL, ydgA, yeaH, yejL, yhgF, yiaf, yicC, yjeB, ymcE*	*E. coli* promoter collection (PEC3877) [35]
E. coli mutant strains	Single gene knockout mutant of *E. coli* K-12 BW25113 including Δ*yddB,* Δ*ampH,* Δ*fliA,* Δ*yidL,* Δ*mutM,* Δ*yjcE,* Δ*yhhS,* Δ*yehL,* Δ*phoH,* Δ*csgF,* Δ*bolA,* Δ*sapA,* Δ*trxC,* Δ*yeaH,* Δ*cspA*	Keio collection [18]
E. coli PHL644/ pMP4655	The plasmid pMP4655 was transformed into *E. coli* K-12 PHL644 strain	This study
S. maltophilia /pBPF-mCherry	The plasmid pBPF-mCherry was transformed into *S. maltophilia* strain	This study
E. coli BW25113 /pMP4655	The plasmid pMP4655 was transformed into *E. coli* K-12 BW25113 strain	This study

Table 5.2 Identified genes that were differentially expressed in mixed species biofilms and planktonic cultures compared to pure *E. coli* biofilms and planktonic cultures and were discussed in this study

Function and gene	Ratio of expression mixed/pure for		Description of gene product
	Biofilms	Planktonic	
Metabolism			
Carbohydrate transport			
araH	4.0	5.0	Fused L-arabinose transport protein
dgoT	6.0	7.3	D-galactonate transporter
rhmT	/	4.9	Predicted L-rhamnonate transporter
vtfR	/	2.5	Putative sugar transport protein
Phosphotransferase			
chbB	/	2.3	N,N'-diacetylchitobiose-specific enzyme IIB
gnd	/	3.0	Gluconate-6-phosphate dehydrogenase
nagE	6.0	8.8	Fused N-acetyl glucosamine specific phosphotransferase
yadI	5.9	5.2	Predicted phosphotransferase enzyme IIA
Ion transport and metabolism			
cysD	4.7	3.6	Sulfate adenylyltransferase subunit 2
fecR	3.3	3.0	Transmembrane signal transducer for ferric citrate transport
ftnA	3.2	/	Ferritin iron storage protein
phnL	3.6	3.4	Phosphate transport
phoH	4.3	/	Protein with nucleoside triphosphate hydrolase domain
pitA	/	2.3	Low-affinity phosphate transport
sirA	3.4	5.3	Sulfurtransferase, sulfur mediator
trxA	/	3.2	Thioredoxin 1
yddB	23.3	23.0	Predicted porin protein
ydeN	3.0	3.0	Putative sulfatase
yfbS	5.1	12.5	Predicted ion transporter
yjcE	8.3	18.4	Predicted cation/proton antiporter

Signal transduction and cell wall component			
Fatty-acid transport			
fadD	3.0	3.1	Long-chain-fatty-acid-CoA ligase
Sensor kinase			
envZ	4.6	3.6	Sensory histidine kinase for OmpR
kdpD	/	2.3	Fused sensory histidine kinase
Peptidoglycan biosynthesis			
ampH	15.7	27.0	Penicillin-binding protein, peptidoglycan synthesis
dacA	/	3.1	D-alanyl-D-alanine carboxypeptidase
ddpA	4.5	4.9	D-ala-D-ala transporter subunit
ddpX	6.8	9.2	D-ala-D-ala dipeptidase
ycfS	3.3	4.5	L,D-transpeptidase linking lipoprotein to murein
Biofilm formation in co-culture			
yddB	23.3	23.0	Predicted porin protein
fliA	15.5	14.4	Flagellar biosynthesis, alternative sigma factor 28
phoH	4.3	/	Protein with nucleoside triphosphate hydrolase domain
sapA	3.3	/	Predicted antimicrobial peptide transporter subunit
cspA	2.5	/	Cold shock protein, transcriptional activator of hns

Note: / indicates non-differential expression of the gene in microarray analysis

Table 5.3 Genes of *E. coli* differentially expressed in biofilms due to co-culture with *S. maltophilia* and differentially expressed due to different growth modes in pure culture

Gene	Relative gene expression		Description of gene product
	Mixed biofilm to pure biofilm	Pure biofilm to pure planktonic	
phoH	4.3	-3.4	Conserved protein with nucleoside triphosphate hydrolase domain
bolA	4.1	-5.6	Transcriptional repressor of mophogene
narZ	3.6	-3.3	Nitrate reductase 2
hspQ	3.1	-2.9	DNA-binding protein, hemimethylated
gadA	2.7	-3.0	Glutamate decarboxylase isozyme
yeaH	2.7	-5.9	Hypothetical protein
rpiR	2.7	-2.4	DNA-binding transcriptional repressor
cspA	2.5	2.9	Cold shock protein, transcriptional activator of hns
ygiM	2.5	-2.4	Predicted signal transduction protein
gadC	2.3	-4.0	Predicted glutamate gamma-aminobutyric acid antiporter
ygeV	-3.2	2.9	Predicted DNA-binding transcriptional regulator

Note: positive value means up-regulation and negative value means down-regulation.

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Appendix 5-1 Full list of identified *E. coli* genes that were differentially expressed in mixed species biofilms with *S. maltophilia* compared to pure *E. coli* biofilms

Gene Name	Fold-Change	Geometric SD	Description of gene product
yddB	23.3	2.8	Predicted porin protein
ampH	15.7	1.9	Beta-lactamase/D-alanine carboxypeptidase
fliA	15.5	2.5	Alternative sigma factor 28, regulation of flagellar operons
yidL	10.3	2.7	Predicted DNA-binding transcriptional regulator
mutM	10.2	3.5	Formamidopyrimidine DNA glycosylase
yjcE	8.3	3.0	Predicted cation/proton antiporter
yhhS	7.5	2.6	Predicted transporter
yigM	7.3	2.4	Predicted inner membrane protein
yebY	7.1	3.0	Predicted protein
ddpX	6.8	2.7	D-ala-D-ala dipeptidase, Zn-dependent
yjhP	6.3	1.9	Putative methyltransferase
dgoT	6.0	2.5	D-galactonate transporter
nagE	6.0	2.7	Fused N-acetyl glucosamine specific PTS enzyme
yadI	5.9	2.8	Predicted PTS enzyme IIA
ymcE	5.5	2.8	Cold shock gene
mrr	5.4	2.1	Methylated adenine and cytosine restriction protein
yjgW	5.3	2.0	KpLE2 phage-like element; predicted protein
purE	5.2	2.4	N5-carboxyaminoimidazole ribonucleotide mutase
yehL	5.1	3.9	Predicted transporter subunit: ATP-binding component of ABC superfamily
yicC	5.1	1.9	Predicted alpha helix protein
yfbS	5.1	2.6	Predicted transporter protein
dhaR	4.9	2.9	Predicted DNA-binding transcriptional regulator, dihydroxyacetone
mhpA	4.9	2.6	3-(3-hydroxyphenyl)propionate hydroxylase
dnaN	4.7	2.0	DNA polymerase III, beta-subunit
cysD	4.7	2.8	Sulfate adenylyltransferase subunit 2
envZ	4.6	2.4	Sensory histidine kinase for OmpR
insB	4.6	2.6	IS1 transposase InsAB
pgpA	4.5	1.7	Phosphatidylglycerophosphatase A
ddpA	4.5	2.5	D-ala-D-a la transporter subunit
insB	4.5	2.8	IS1 protein InsB

phoH	4.3	2.7	Conserved protein with nucleoside triphosphate hydrolase domain
sfsB	4.2	3.1	DNA-binding transcriptional activator of maltose metabolism
csgF	4.2	1.9	Curli production assembly/transport component, 2nd curli operon
bolA	4.1	1.9	Transcriptional repressor of mophogene
araH	4.0	2.1	Fused L-arabinose transport protein (ABC superfamily)
yfjF	4.0	2.8	Predicted protein
fixX	3.9	2.7	Predicted ferredoxin-type protein
exoD	3.9	2.8	Pseudogene, exonuclease,bacteriophage
wcaB	3.8	2.6	Predicted acyl transferase
rfaB	3.7	2.3	*UDP-D*-galactose:(glucosyl)lipopolysaccharide-1, 6-D-galactosyltransferase
helD	3.7	2.0	DNA helicase IV
bcsG	3.7	2.1	Predicted inner membrane protein
narZ	3.6	2.0	Cryptic nitrate reductase 2
phnL	3.6	1.7	ATP-binding component of phosphonate transport
hisI	3.6	2.4	Phosphoribosyl-amp cyclohydrolase; phosphoribosyl-ATP pyrophosphatase
yjhD	3.6	2.2	Orf, hypothetical protein
uvrC	3.5	2.1	Excinuclease ABC, subunit C; repair of UV damage to DNA
frlB	3.5	2.1	Fructoselysine-6-P-deglycase
cspB	3.5	2.0	Qin prophage; cold shock protein
rimI	3.4	1.9	Acyltransferase for 30S ribosomal subunit protein S18
sirA	3.4	2.0	Conserved protein required for cell growth
yejL	3.4	2.2	Orf, hypothetical protein
panF	3.4	2.2	Sodium/pantothenate symporter
wcaK	3.4	2.3	Predicted pyruvyl transferase
yghJ	3.4	2.2	Predicted inner membrane lipoprotein
ycfS	3.3	2.0	L,D-transpeptidase linking Lpp to murein
fecR	3.3	1.8	KpLE2 phage-like element; transmembrane signal transducer for ferric citrate transport
sapA	3.3	1.8	Predicted antimicrobial peptide transporter subunit
kilR	3.3	1.6	Rac prophage, inhibitor of ftsZ, killing protein
acpS	3.3	2.3	Holo-[acyl-carrier-protein] synthase 1
phnP	3.2	1.8	Carbon-phosphorus lyase complex protein

tdcD	3.2	2.5	Propionate kinase/acetate kinase C
rsgA	3.2	1.8	Ribosome small subunit-dependent GTPase A
ftn	3.2	1.7	Cytoplasmic ferritin iron storage protein
nuoE	3.1	2.5	NADH ubiquinone oxidoreductase chain E
bhsA	3.1	1.7	Orf, hypothetical protein
hspQ	3.1	2.3	DNA-binding protein, hemimethylated
fadD	3.0	2.4	Acyl-CoA synthetase, long-chain-fatty-acid--CoA ligase
ydeN	3.0	2.3	Putative sulfatase
ydgA	3.0	2.1	Orf, hypothetical protein
yhgF	2.9	1.8	Predicted transcriptional accessory protein
yfaV	2.8	1.7	Putative transport protein
rutA	2.7	1.8	Predicted monooxygenase
gadA	2.7	1.6	Glutamate decarboxylase isozyme
trxC	2.7	1.6	Thioredoxin 2
yeaH	2.7	1.7	Orf, hypothetical protein
rpiR	2.7	1.6	DNA-binding transcriptional repressor
ycgL	2.6	1.7	Orf, hypothetical protein
cspA	2.5	1.7	Cold shock protein 7.4, transcriptional activator of hns
ygiM	2.5	1.4	Predicted signal transduction protein (SH3 domain)
yqhD	2.5	1.3	Alcohol dehydrogenase, NAD(P)-dependent
yefM	2.3	1.3	Antitoxin of the YoeB-YefM toxin-antitoxin system
gadC	2.3	1.3	Predicted glutamate gamma-aminobutyric acid antiporter
corA	-2.4	1.4	Magnesium/nickel/cobalt transporter
intR	-2.6	1.5	Rac prophage, integrase
rsxG	-2.8	1.5	Predicted oxidoreductase
rbsB	-2.9	1.8	D-ribose transporter subunit
yicG	-3.1	1.6	Conserved inner membrane protein
ygeV	-3.2	1.5	Predicted DNA-binding transcriptional regulator

Note: Positive fold-change value is (gene expression in mixed species biofilms)/(expression in pure biofilms). Negative fold-change value is -(gene expression in pure biofilms)/(gene expression in mixed species biofilms). The value is mean of two biological replicates, each with two technical replicates.
Geometric SD is 2^{SD}, where SD is standard deviation of $\log_2$ transformation of fold-change in each replicate. Differentially expression means fold-change over 2 with one sample single tail t-test p-value less than 0.05.

Appendix 5-2 Full list of identified *E. coli* genes that were differentially expressed in mixed species planktonic cultures with *S. maltophilia* compared to *E. coli* pure planktonic cultures

Gene Name	Fold-Change	Geometric SD	Description of gene product
ampH	27.0	3.2	Beta-lactamase/D-alanine carboxypeptidase
yddB	23.0	3.5	Predicted porin protein
gsp	19.3	1.5	Glutathionylspermidine synthetase/amidase
yjcE	18.4	4.5	Predicted cation/proton antiporter
fliA	14.4	3.4	alternative sigma factor 28; regulation of flagellar operons
mutM	12.6	3.3	Formamidopyrimidine DNA glycosylase
yfbS	12.5	2.2	Predicted transporter protein
yicC	11.0	2.2	Predicted alpha helix protein
yhhS	10.8	1.9	Predicted transporter
yjhP	10.8	1.8	Putative methyltransferase; KpLE2 phage-like element
purE	10.0	3.3	N5-carboxyaminoimidazole ribonucleotide mutase
ddpX	9.2	2.5	D-ala-D-ala dipeptidase, Zn-dependent
ybjD	9.2	1.7	Conserved protein with nucleoside triphosphate hydrolase domain
nagE	8.8	4.1	Fused N-acetyl glucosamine specific PTS enzyme; contribute to peptidoglycan recycle
yidL	8.4	2.1	Predicted DNA-binding transcriptional regulator
mrr	8.4	2.1	Methylated adenine and cytosine restriction protein
yigM	8.0	1.8	Predicted inner membrane protein
dgoT	7.3	2.2	D-galactonate transporter
yebY	7.1	2.7	Predicted protein
dnaN	7.1	1.7	DNA polymerase III, beta-subunit
yjgW	6.4	1.7	KpLE2 phage-like element; predicted protein
uvrC	6.2	2.8	Excinuclease ABC, subunit C; repair of UV damage to DNA
sfsB	6.1	1.7	DNA-binding transcriptional activator of maltose metabolism
nrfG	5.9	2.6	Part of formate-dependent nitrite reductase complex
ymcE	5.7	2.2	Cold shock gene
yejL	5.3	2.2	Orf, hypothetical protein
yrhB	5.3	2.3	Orf, hypothetical protein

sirA	5.3	1.9	Conserved protein required for cell growth
pgpA	5.2	1.6	Phosphatidylglycerophosphatase A
yadI	5.2	1.6	Predicted PTS enzyme IIA
yhjU	5.1	2.5	Predicted inner membrane protein
insB	5.1	2.2	IS1 transposase InsAB
araH	5.0	1.5	Fused L-arabinose transport protein (ABC superfamily)
yfaV	4.9	1.9	Putative transport protein
ddpA	4.9	2.4	D-ala-D-a la transporter subunit
ydgA	4.8	2.8	Orf, hypothetical protein
insB	4.6	1.9	IS1 protein InsB
yjhD	4.5	1.9	Orf, hypothetical protein
ycfS	4.5	2.3	L,D-transpeptidase linking Lpp to murein
yghJ	4.4	1.6	Predicted inner membrane lipoprotein
mhpA	4.4	2.4	3-(3-hydroxyphenyl)propionate hydroxylase
rfaB	4.4	2.0	*UDP-D*-galactose:(glucosyl)lipopolysaccharide-1, 6-D-galactosyltransferase
dhaR	4.4	2.0	Predicted DNA-binding transcriptional regulator, dihydroxyacetone
fixX	4.2	1.5	Predicted ferredoxin-type protein
nuoE	4.2	2.2	NADH ubiquinone oxidoreductase chain E
cspB	4.2	2.2	Qin prophage; cold shock protein
wcaB	4.1	2.8	Predicted acyl transferase
hisI	4.0	1.6	Phosphoribosyl-amp cyclohydrolase; phosphoribosyl-ATP pyrophosphatase
rimM	4.0	1.9	16S rRNA-processing protein
acpP	3.8	1.4	Putative acyl carrier protein
yhgF	3.8	1.3	Predicted transcriptional accessory protein
wcaK	3.8	1.6	Predicted pyruvyl transferase
acpS	3.8	1.5	Holo-[acyl-carrier-protein] synthase 1
insZ	3.8	1.8	Orf, hypothetical protein
ycgL	3.7	1.8	Orf, hypothetical protein
alaS	3.7	1.7	Alanyl-tRNA synthetase
ynfG	3.6	1.6	Putative oxidoreductase Fe-S subunit
envZ	3.6	1.9	Sensory histidine kinase for OmpR
yfiR	3.6	1.9	Orf, hypothetical protein
cysD	3.6	1.7	Sulfate adenylyltransferase subunit 2
pmrD	3.5	1.6	Polymyxin resistance protein B
exoD	3.5	1.8	Pseudogene, exonuclease,bacteriophage

ykfC	3.4	2.0	Orf, hypothetical protein
yhdW	3.4	2.1	Putative periplasmic binding transport protein
phnL	3.4	1.6	ATP-binding component of phosphonate transport
insB	3.4	2.1	IS1 protein InsB
insB	3.3	1.9	IS1 transposase InsAB
pyrD	3.3	2.2	Dihydro-orotate oxidase
trxA	3.2	1.5	Thioredoxin 1
ybeY	3.2	1.5	Conserved protein
pntA	3.2	1.0	Pyridine nucleotide transhydrogenase, alpha subunit
nsrR	3.1	1.6	Predicted DNA-binding transcriptional regulator
rimI	3.1	1.4	Acyltransferase for 30S ribosomal subunit protein S18
fadD	3.1	1.4	Acyl-CoA synthetase, long-chain-fatty-acid--CoA ligase
dacA	3.1	1.6	D-alanyl-D-alanine carboxypeptidase; penicillin-binding protein 5
fecR	3.0	1.3	KpLE2 phage-like element
gnd	3.0	1.6	Gluconate-6-phosphate dehydrogenase, decarboxylating
helD	3.0	1.7	DNA helicase IV
ydeN	3.0	2.0	Putative sulfatase
fliS	3.0	1.3	Serine/threonine-specific protein phosphatase 1
yfjF	2.9	1.9	Predicted protein
prr	2.9	1.9	Medium chain aldehyde dehydrogenase
rzpR	2.9	1.6	Putative Rac prophage endopeptidase
folA	2.9	1.4	Dihydrofolate reductase
rsgA	2.9	1.5	Ribosome small subunit-dependent GTPase A
insB	2.8	1.8	InsB IS1 transposase
wzc	2.8	1.8	Protein-tyrosine kinase
bhsA	2.8	1.7	Orf, hypothetical protein
tdcD	2.7	1.6	Propionate kinase/acetate kinase C
aceF	2.6	1.6	Pyruvate dehydrogenase, dihydrolipoyltransacetylase component
phnP	2.6	1.2	Carbon-phosphorus lyase complex accessory protein
cpxP	2.6	1.3	Periplasmic protein combats stress
ytfR	2.5	1.5	Putative sugar transport protein

crl	2.5	1.3	DNA-binding transcriptional regulator
yiaF	2.5	1.5	Orf, hypothetical protein
pphA	2.5	1.4	Serine/threonine-specific protein phosphatase 1
pmbA	2.5	1.4	Predicted peptidase
yddL	2.4	1.4	Putative outer membrane porin protein
ydcU	2.4	1.4	Putative spermidine/putrescine transporter subunit
ygdB	2.4	1.4	Orf, hypothetical protein
kdpD	2.3	1.3	Fused sensory histidine kinase
pitA	2.3	1.3	Low-affinity phosphate transport
chbB	2.3	1.3	N,N'-diacetylchitobiose-specific enzyme IIB component of PTS
mglC	-2.4	1.4	Methyl-galactoside transporter subunit
rsxG	-2.5	1.5	Predicted oxidoreductase
gadC	-2.7	1.8	Predicted glutamate gamma-aminobutyric acid antiporter
gadA	-2.8	1.5	Glutamate decarboxylase isozyme
ygeV	-2.9	1.6	Predicted DNA-binding transcriptional regulator

Note: Positive fold-change value is (gene expression in mixed species planktonic cultures)/(gene expression in pure planktonic cultures). Negative fold-change value is (gene expression in pure planktonic cultures)/(gene expression in mixed species planktonic cultures). The value is mean of two biological replicates, each with two technical replicates.

Geometric SD is 2^{SD}, where SD is standard deviation of $\log_2$ transformation of fold-change in each replicate. Differentially expressed means fold-change over 2 with one sample single tail t-test p-value less than 0.05.

Chapter 6
Gene *fliA* Mediates the Inhibition of Biofilm Formation by *Escherichia coli* when Interacts with *Stenotrophomonas maltophilia*

6.1 Abstract

Our previous study found that biofilm formation of *Escherichia coli* PHL644 was significantly inhibited in mixed species culture with *Stenotrophomonas maltophilia* (Chapter 3). Transcriptomic study identified that gene *fliA* was among the most highly induced genes of *E. coli* in co-culture with *S. maltophilia* compared with that in single species culture (Chapter 5). This study investigated the role of *fliA* in species interactions and inhibited biofilm formation by *E. coli* in mixed species culture. Induction of *fliA* expression was confirmed as resulting from direct cell-to-cell interactions between *E. coli* and *S. maltophilia,* rather than due to nutrition depletion in co-culture with *S. maltophilia*. Overexpression of *fliA* slightly increased cell motility of the non-motile strain *E. coli* PHL644. However, *fliA* overexpression significantly reduced cell aggregation and the production of a major curli monomer protein CsgA. Cell appendage curli was previously demonstrated as critical for cell aggregation and therefore biofilm formation of *E. coli* PHL644 in mono-species culture. Therefore, it was concluded that up-regulation of *fliA* in *E. coli* when interacting with *S. maltophilia* lead to reduced curli production and resulting inhibited biofilm formation of *E. coli* in mixed species culture.

6.2 Introduction

Biofilms in nature are usually mixed-species consortia, in which various species interactions exist and affect the composition of biofilms [1]. Our previous study found that interactions between *Escherichia coli* and *Stenotrophomonas maltophilia* inhibited biofilm development by *E. coli* in static batch culture (Figure 3.2). It also showed that cell aggregation and surface attachment of *E. coli* was reduced when mixing with *S. maltophilia* cells. Transcriptomic study has identified a list of *E. coli* gene differentially

expressed when *E. coli* was co-cultured with *S. maltophilia* compared with *E. coli* in mono-species culture. Gene *fliA* was among the most highly induced genes, with a 15-fold induction in expression when *E. coli* was in mixed species culture (Table 5.2). The purpose of this study is to examine the role of induced gene *fliA* in inhibited *E. coli* biofilm formation in mixed culture.

FliA is an alternative sigma factor (σ^{28}) responsible for the initiation of transcription of several genes involved in cell motility. Most notable are for flagellar biosynthesis and functions, such as *fliC* encoding flagellin and *motAB* encoding stator element of the flagellar motor complex [2]. Expression of gene *fliA* itself is under the regulation of a master gene *flhDC,* which is regulated by quorum-sensing regulon *qseBC* in some *E. coli* strain [3]. Biofilm formation of *E. coli* in pure culture could be affected by the expression of these motility genes (*fliA, fliC, motA, flhD,* and *qseB*) [4, 5].

The *E. coli* PHL644 strain used in our previous studies was a derivative of *E. coli* MC4100, which carried a +1 frameshift in gene *flhDC* mutation [6, 7]. Expression of *fliA* was shown as very low in this strain [6]. But the strain PHL644 was previously screened out as a robust biofilm former, which had high expression of curli resulting high aggregation and biofilm formation in microtiter plates [7]. Inverse regulation of motility and curli mediated cell adhesion was previously reported [6, 8]. It was interesting to investigate relationship of *fliA* upregulation and curli production with regard to reduced cell aggregation and biofilm formation of *E. coli* in co-culture with *S. maltophilia.*

This study firstly confirmed *fliA* upregulation in *E. coli* as due to direct contact with *S. maltophilia* cells. The mechanisms of *fliA*-mediated alteration in biofilm formation of *E. coli* in co-culture were further examined.

6.3 Results

6.3.1 Induction of *fliA* required cell-to-cell contact in mixed species culture

Up-regulation of *E. coli* gene *fliA* in mixed species culture with *S. maltophilia* was identified in previous transcriptomic study (Table 5.2). Its induction was confirmed in the same culture condition as used for the transcriptomic study (18 h culture at room

temperature, early stationary stage) using an *E. coli* transcription reporter strain *E. coli/pfliA-gfp*, which carried a green fluorescence protein (*gfp)* gene under the control of *fliA* promoter (Figure 5.3). Expression of *fliA* was further tested in different culture conditions to confirm that its up-regulation actually resulted from species interactions.

Extended growth of *E. coli/pfliA-gfp* from early to late stationary phase (18 h to 24 h) in both pure and mixed species cultures were conducted to test the effect of nutrient depletion on *fliA* expression. Cells in later growth phase encounter more serious depletion of substrate than in earlier growth phase. The result showed that expression of *fliA* in mixed species culture at earlier growth phase (e.g. 18 h) was higher than that in mono-species culture at later stationary phase (e.g. 24 h). (Figure 6.1A).

The effect of physical cell-to-cell contact between *E. coli* and *S. maltophilia* on gene *fliA* expression was tested using the Transwell system. *E. coli/pfliA-gfp* and *S. maltophilia* were cultured together but separated (without direct cell-contact between the two species) by porous membrane in a Transwell system. The results (Figure 6.1B) showed that *fliA* expression of *E. coli* in the contact-free was similar to the level in pure *E. coli* culture; both significantly lower than that in mixed species culture with *S. maltophilia.*

6.3.2 Gene *fliA* affects *E. coli* biofilm formation in mixed species culture

The effect of *fliA* expression on biofilm formation by *E. coli* was examined with three *E. coli* strains, wild type BW25113 strain*,* knockout mutant Δ*fliA,* and PHL644/pCA24N-*fliA* strain with inducible *fliA* expression. Single and mixed species biofilms with *S. maltophilia* were cultured in static microtiter plates.

Biomass of the biofilm by knockout mutant Δ*fliA* was not significantly different from that of wild type *E. coli* BW25113 (Figure 5.4B), while the structure of biofilms looked differently. Wild type biofilms had more cell aggregates and Δ*fliA* biofilm cells were more evenly spread on surface (Figure 6.2A,D). Biofilm formation by *E. coli* wild type and Δ*fliA* mutant strains was both inhibited when co-cultured with *S. maltophilia.* However, the inhibition was weakened when gene *fliA* was knocked out. Wild type *E. coli* biofilm formation was greatly inhibited by *S. maltophilia*, with few *E. coli* cells

grown as biofilm, even if on the surface close to the edge of wells where more attached cells were usually observed (Figure 6.2B,C). In contrast, more Δ*fliA* cells could attach to the surface in mixed species culture (Figure 6.2E), especially near the edge of microtiter wells (Figure 6.2F).

Expression of *fliA* in *E. coli*/pCA24N-*fliA* could be induced when chemical IPTG (isopropyl β-D-1-thiogalactopyranoside) was supplemented in culture media. Biofilm formation by *E. coli*/pCA24N-*fliA* was significantly reduced when *fliA* was overexpressed in single species culture (Figure 6.2G,H). However, growth inhibition of *E. coli*/pCA24N-*fliA* cells was also observed when *fliA* expression was induced by 50 μM IPTG (data not shown).

6.3.3 Increased cell motility with overexpressed *fliA*

E. coli PHL644 was a non-motile strain due to a mutation in gene *flhDC,* which was the master regulator of *fliA*. Expression of *fliA* was very low in *E. coli* MC4100, the parental strain of PHL644. Several flagella biosynthesis gene (e.g. *fliC, motA*) were under the control of gene *fliA*. External induction of *fliA* by IPTG may induce expression of these genes and flagella biosynthesis, resulting increased cell motility. Cell motility of *E. coli* PHL644 was thus examined on soft agar when gene *fliA* was induced by IPTG. The results showed that swimming ability of *E. coli*/pCA24N-*fliA* was increased (colony diameter increased from 6.5 mm to 8.5 mm) when 5 or 50 μM IPTG was supplemented in media (Figure 6.3). Swarming and twitching ability of *E. coli*/pCA24N-*fliA* showed no difference whether IPTG was supplemented or not (results not shown).

6.3.4 Decreased cell aggregation and curli production with induced *fliA*

Thick biofilm formation of *E. coli* PHL644 in pure culture was showed as a result of increased curli production and cell aggregation [7]. To test whether induced expression of *fliA* in mixed species culture caused inhibited biofilm formation of *E. coli,* the effects of *fliA* induction on cell aggregation and on curli production were examined.

Cell aggregation was shown as a percentage of cells forming aggregates and settling down, resulting in decreased optical density in the top layer of a culture column. *E. coli*

and *E. coli*/pCA24N-*fliA* were cultured in media with 0 or 50 μM IPTG for aggregation test. Results showed that aggregation of *E. coli*/pCA24N-*fliA* was significantly reduced when cells were cultured in media with 50 μM IPTG (Figure 6.4). Supplement of IPTG showed no effect on cell aggregation of *E. coli* itself (Figure 6.4).

Effect of *fliA* induction on curli production was examined using *E. coli* PHL644 strain with inducible *fliA* expression (*E. coli*/pTOPO-*fliA*) and corresponding vector control strain *E. coli*/pTOPO, cultured in media supplemented with inducer chemical anabinose at concentrations of 0, 0.02% and 0.2%. Curli production in the two strains was estimated through two critical curli proteins, CsgA and CsgG. CsgA is a major curli subunit. CsgG is an outer membrane-located protein for the secretion of CsgA. Production of CsgA and CsgG was semi-quantified with whole-cell western blot assay with normalized cell loading. The results (Figure 6.5) showed production of CsgA in *E. coli* and the vector control strain *E. coli*/pTOPO in three cultures with/without arabinose. However, *E. coli*/pTOPO-*fliA* cells showed very weak or no production of CsgA in all tested cultures. In contrast, protein CsgG was produced in all three tested strains (Figure 6.5).

6.4 Discussion

Our previous study found that *E. coli* biofilm formation was significantly inhibited in static mixed species culture with *S. maltophilia* (Figure 3.2). Gene *fliA* of *E. coli* was identified as highly up-regulated when *E. coli* was co-cultured with *S. maltophilia* by cDNA microarray analysis (Table 5.2). This study confirmed that induction of *fliA* was resulted from species interactions and investigated how *fliA* upregulation affected biofilm formation by *E. coli.*

6.4.1 Induction of gene *fliA* resulted from species interactions

Induction of *fliA* expression was confirmed using *fliA* transcription reporter strain *E. coli/pfliA-gfp* cultured at the same conditions as used for cDNA microarray analysis, which was 18-h planktonic culture or biofilm in 0.1×LB at room temperature. Both pure culture of *E. coli* and mixed species culture of *E. coli* and *S. maltophilia* reached early stationary phase in growth curves after 18-h growth (data not shown). In mixed species batch culture, one species compete for limited substrate with other species. Nouaille et al.

found that *Lactococcus lactis* encountered earlier carbon starvation in mixed species cultures than in its single species culture [9]. Mover, they found that many genes differentially expressed in mixed species culture were due to nutrition competition and showed similar expression trends in single species culture in later growth phase [9]. So we first tested whether up-regulation of *fliA* was due to nutritional limitation encountered by *E. coli* in mixed species culture. Growth of *E. coli/pfliA-gfp* in single and mixed species cultures were extended from 18 h to 24 h, reaching late stationary phase. Expression of *fliA* in mono-species pure culture was low and decreased a little with growth (Figure 6.1A). Gradually shutting down flagella genes after exponential growth phase was reported previously [10]. However, it was found that the expression of *fliA* in mixed species cultures at earlier growth phase (18 h) were always higher than that in single species cultures at all tested time points (every one hour during 18 and 24 h, Figure 6.1A). It suggested that *fliA* induction was due to species interactions, rather than early nutrition depletion due to species competition in mixed species culture.

Our previous study (Figure 3.3B) found that cell-to-cell contact between the two species was required primarily for inhibition of *E. coli* biofilm formation in mixed species cultures. With mixed species cultures in a Transwell system to prevent direct cell-contact between the two species, we found that *fliA* up-regulation also required cell-contact between *E. coli* and *S. maltophilia* (Figure 6.1B). It further confirmed that induction of *fliA* resulted from species interactions. It was thus interesting to study the relationship of up-regulation of *fliA* with reduced *E. coli* biofilm formation in mixed species cultures.

6.4.2 Gene *fliA*, cell motility and biofilm formation

Master regulator gene *flhDC*, sigma factor gene *fliA,* and flagellin and assembly gene *fliCD, motAB* etc. are in hierarchy transcription in flagellar synthesis and functions. Gene *fliA* plays a critical role in bacterial flagellar, which is an important rotary machine required for cell motility. Knockout mutants of *fliA* for several species (e.g. *E. coli, Legionella pneumophila*) showed decreased or aborted cell motility in previous studies [4, 11]. Expression of *fliA* in strain *E. coli* PHL644 used in this study was very low, due to a mutation in gene *flhDC* [12]. Low cell motility of this strain was confirmed in this study (Figure 6.3). Although with a nonfunctional master regulator FlhD, overexpression

of *fliA* from an external plasmid may be able to initiate transcription of flagella class III genes [8], resulting in restored, though very little, cell motility as observed in Figure 6.3.

Previous studies showed that gene *fliA* was involved in biofilm formation through affected flagellar function and cell motility [4]. Knockout mutants of *fliA* for several species, such as *E. coli* MG1655*, Legionella* and *Salmonella spp.* showed less biofilm formation than wild type strains [4, 11, 13]. However, there is controversy about the effect of flagellar and cell motility on biofilm formation since the effects varied among species [14]. In this study, biomass of single specie biofilm by Δ*fliA* mutant was not significantly different from that of wild type *E. coli* BW25113 (Figure 5.4B), while the structure of biofilms showed some difference (Figure 6.2A,D). The results suggested that gene *fliA,* or flagella and cell motility may be not critical for biofilm formation by the strain BW25113.

However, gene *fliA* did show its impact on biofilm formation of *E. coli* in mixed species cultures with *S. maltophilia*. The inhibition of *S. maltophilia* on *E. coli* biofilm formation in co-culture was diminished when gene *fliA* was knocked out (Figure 6.2B,C,E,F). It was confirmed in this study that gene *fliA* was induced as a result of species interactions. Knocking out *fliA* would cut off partial interactions and thus mitigate the negative effect of species interactions on biofilm formation. Although cell motility of *E. coli* increased with overexpressed *fliA*, the increase was very limited (Figure 6.3) and the strain *E. coli*/pCA24N-*fliA* was non-motile. It is likely that the effect of *fliA* on biofilm formation of *E. coli* may be through other mechanisms rather than flagellar or cell motility.

It was previously found that flagellar was not required for biofilm development in curli-overexpression strain [15]. *E. coli* strain PHL644 was a curli-overexpression strain. Moreover, curli production was actually the reason of robust biofilm formation by this strain in pure culture [7]. So the effect of *fliA* overexpression on curli production for this strain was further explored.

6.4.3 Inverse effect of *fliA* induction on expression of curli gene *csgA*

Curli was found as adhesive fimbriae of several species including *E. coli.* Curli is involved in cell aggregation, adhesion to host cells, and biofilm formation [16]. High cell

aggregation (91.5-93.4% Figure 6.4) of *E. coli* PHL644 was a result of curli overexpression. Reduced cell aggregation (72.3%) due to *fliA* overexpression (Figure 6.4) suggested a relationship between *fliA* overexpression and curli production.

In *E. coli,* curli production involves at least six proteins. CsgA and CsgB are the major and miner monomer of curli fibers, respectively. CsgD is a transcription activator of *csgBA*. CsgGEF are nonfiber structural proteins for secretion of monomer (CsgG) and stabilization and modification of curli assembly (CsgEF) [17]. The effect of *fliA* overexpression on curli production was thus investigated by measuring the production of two of these curli proteins, CsgA and CsgG. Results showed little production of CsgA and uninfluenced CsgG synthesis in the *fliA* overexpression strain *E. coli/*pTOPO-*fliA* (Figure 6.5). Monomer CsgA is definitely critical for curli assembly. Curli may not assemble with little production of CsgA. Thus it was reasonable to infer that curli production was inhibited by *fliA* overexpression, resulted in reduced cell aggregation as observed in Figure 6.4.

Inverse regulation of *fliA* and curli during exponential and stationary phase in *E. coli* was reported previously [10]. Expression of curli genes was under the control of stationary phase sigma factor (σ^{38}). Since gene *fliA* encodes another sigma factor, σ^{28}, overexpressed σ^{28} may outcompete σ^{38} for RNA polymerase when *fliA* expression was induced externally, resulting inhibited curli production. Pesavento et al. found that *fliA* induction could repress signaling system via c-di-GMP (bis-(3´-5´)-cyclic-diguanosine monophosphate), which actually initiated transcription of curli genes [10]. Observed negative effect on CsgA production by *fliA* overexpression was thus understandable, although why CsgG production was not affected needed further investigation.

6.5 Conclusions

Our previous study found that a robust biofilm former strain, *E. coli* PHL644, was significantly inhibited in biofilm formation in co-culture with *S. maltophilia.* Gene *fliA* in *E. coli* was identified as highly induced in co-cultures. This study confirmed that the induction of *fliA* was due to species interactions with *S. maltophilia,* rather than substrate depletion resulted from nutrient competition between *E. coli* and *S. maltophilia* in mixed

species cultures. Overexpression of *fliA* only slightly affected cell motility of *E. coli* PHL644, but significantly inhibited production of critical curli protein CsgA and cell aggregation. It was concluded that reduced curli production of *E. coli* PHL644 due to induced *fliA* expression by species interactions was responsible for inhibited biofilm formation of *E. coli* in mixed species cultures.

Our study found that cell-to-cell contact between *E. coli* and *S. maltophilia* was required both in induction of *fliA* and in inhibited biofilm formation of *E. coli* in co-cultures. Curli and flagella are bacterial surface components and are reported to be involved in cell-to-cell interactions. How *S. maltophilia* induce the expression of *E. coli* gene *fliA* by cell-to-cell contact between the two species warrants further investigation in future study.

6.6 Materials and Methods

6.6.1 Bacterial strains and cultures

All bacterial strains used in this study were listed in Table 6.1. All strains were grown on Luria Broth (LB) agar or 10-fold diluted LB broth (0.1×LB) supplied with appropriate antibiotics (Sigma-Aldrich, St. Louis, MO): tetracycline (20 μg/ml), gentamicin (20 μg/ml), kanamycin (25 μg/ml), ampicillin (50 μg/ml) or chloramphenicol (30 μg/ml). No antibiotics were supplied in mixed species cultures. Planktonic cultures were incubated with continuous shaking (250 rpm) at 30 °C for 15 h.

6.6.2 Biofilm culture and imaging

Biofilms were grown in static glass-bottom 24-well plates (MatTek, Ashland, MA) containing 1 ml broth/well at room temperature (20°C) for 24 h. Inoculation was 1:100 (v/v) from overnight cultures with a final concentration around 5×10^6 CFU/ml. Ratio of *E. coli* and *S. maltophilia* was adjusted to around 1:1 in mixed-species biofilm formation.

To image biofilms, suspended cells in each well were removed and biofilms were gently washed three times with 1×PBS (phosphate buffered saline). Biofilms formed in the center of each well, except specifically indicated, were randomly chosen for imaging with a confocal laser scanning microscopy (CLSM) (Olympus, Center Valley, PA) equipped with the software FluoView 300 (Olympus, Center Valley, PA). Most *E. coli* strains and

S. maltophilia used in this study were labeled with fluorescent proteins (Table 6.1). Non-labeled *E. coli*/pCA24N-*fliA* was stained with 5 μM SYTO 9 (Invitrogen, Carlsbad, CA) according to manufacture's protocol. A series of CLSM images was taken every 1 μm in the depth of biofilm and combined to reconstruct 3-D biofilms using the software Volocity 3.2 (PerkinElmer, Fremont, CA).

6.6.3 Confirmation of *fliA* expression

Differential expression of *fliA* in mixed species cultures was confirmed with *E. coli* reporter strain, which carried a *gfp* gene in the control of promoter of gene *fliA*. Fluorescence intensity of the reporter strain thus indicated expression of *fliA*. Cell contact-free mixed-species cultures were performed in Transwell systems (Corning, Lowell, MA), with *E. coli* in 24-well microtiter plates and *S. maltophilia* grown in the inserts. *E. coli* reporter strain grown in planktonic pure cultures, mixed-species cultures, and in Transwells were analyzed with flow cytometry as previously described (Chapter 5).

6.6.4 Cell motility test

Media used to test swimming ability contained 3 g/l agarose, 10 g/l tryptone, and 5 g/l sodium acetate with 0, 5 or 50 μM IPTG (Fermentas, Glen Burnie, MD). Inoculation was from fresh culture in LB agar. Plates were cultured at 30 °C for 24 h.

6.6.5 Cell aggregation assay

E. coli and *E. coli*/pCA24N-*fliA* were grown in 0.1×LB, supplied with appropriate antibiotics, for four hours to allow initial growth of cells before IPTG (50 μM) was supplemented. Cells in the overnight cultures were re-suspended in fresh 0.1×LB and adjusted to similar optical density (OD at 600 nm). Cultures in test tubes (3 ml/tube) were set static on bench for 24 h. Optical density of cultures in the top layer was measured and cell aggregation was expressed as the relative decrease of OD.

6.6.6 Whole-cell western analysis assay

Planktonic cultures of *E. coli, E. coli/*pTOPO, *E. coli*/pTOPO-*fliA* and Δ*csgA* were grown in 0.1×LB supplied with appropriate antibiotics and/or inducer arabinose (0.2% or 0.02%) for overnight at 30 °C. Cell density of each culture was adjusted to OD of 0.2.

Pellets of bacterial cells from 1 ml cultures were treated with 75 μl hexafluoro-2-propanol (HFIP). HFIP was removed by spinning in a vacuum for 35 min at 42 °C and treated cells were resuspended in 150 μl 2× SDS loading buffer. Samples were separated on a 15% SDS-polyacrylamide gel and blotted onto polyvinylidene fluoride membrane (Fisher Scientific, Pittsburgh, PA) with standard method [18]. The blot was firstly probed with anti-CsgA polyclonal antibody (1:10,000) and a secondary anti-rabbit antibody (1:7,000) used by Chapman et al. The blot was developed using the Pierce SuperSignal duration substrate (Fisher Scientific) as described previously [18]. The same blot was then stripped with stripping buffer (Fisher Scientific), probed with anti-CsgG antibody (1:100,000) and the second antibody, and developed as above.

6.7 Figures and Tables

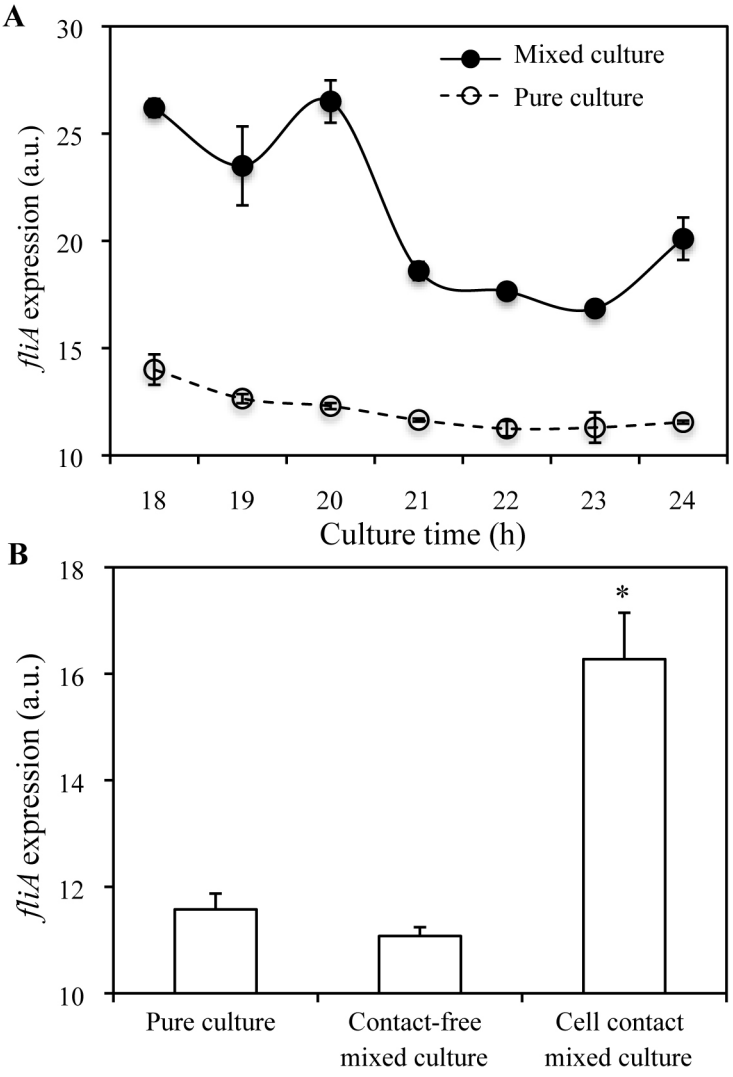

Figure 6.1 Confirmed up-regulation of *fliA* in mixed species cultures

Expression of *fliA* in reporter strain *E. coli/pfliA-gfp* (A) during extended growth in planktonic single and mixed species cultures (18-24 h after inoculation) and (B) in pure culture, contact-free mixed species culture, and mixed species co-culture with *S. maltophilia*. *fliA* expression was shown as fluorescence intensity in arbitrary unit (a.u.) of a population of 30,000 *E. coli/pfliA-gfp* cells measured by flow cytometry. Contact-free mixed culture was conducted in Transwell systems with *S. maltophilia* in inserts and *E. coli* in the corresponding wells. Two independent replicates were conducted for each culture. Error bars show standard deviations of replicates. Symbol * indicates significant difference ($p<0.05$) using pure culture as a control.

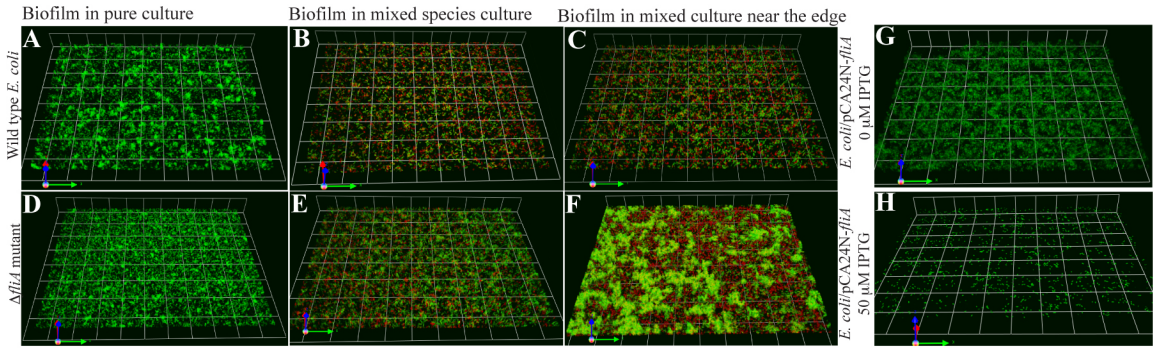

Figure 6.2 Effect of *fliA* knockout on *E. coli* biofilm formation in single and mixed species culture

Three dimensional images of biofilms by wild type *E. coli* BW25113 (A,B,C) and its knockout mutant strain Δ*fliA* (D,E,F) in single species pure cultures (A,D) and in mixed species cultures with *S. maltophilia* (B,C,E,F) and biofilm formation of *E. coli*/pCA24N-*fliA* cultured in media supplemented with (G) 0 μM and (H) 50 μM IPTG. Biofilms were all culture in 0.1×LB broth in static glass-bottom microtiter plates for 22 h at room temperature. *E. coli* wild type and *fliA* knockout carried a plasmid pMP4655 and shown as green, and *S. maltophilia* carried a plasmid pBPF-mCherry and shown as red. *E. coli*/pCA24N-*fliA* biofilm were stained with SYTO-9 and shown as green. 3-D images were compiled from a stack of confocal laser scanning microscopic images, which were randomly taken over biofilms grown in the center of wells except C and F. Grid size is 26.8 μm.

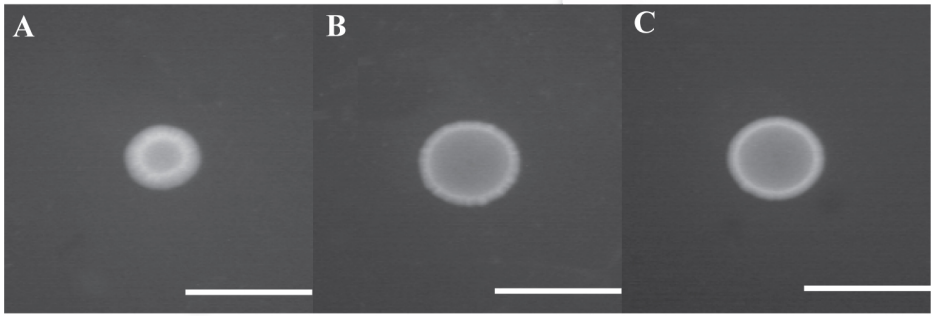

Figure 6.3 Effect of *fliA* expression on cell swimming ability of *E. coli* PHL644

Swimming test of *E. coli*/pCA24N-*fliA* strain on soft agar plate (0.3% agarose) supplemented with (A) 0 μM (B) 5 μM and (C) 50 μM IPTG, which induced the expression of gene *fliA*. The diameter of colonies shows the ability of cell swimming. Scale bars are 10 mm.

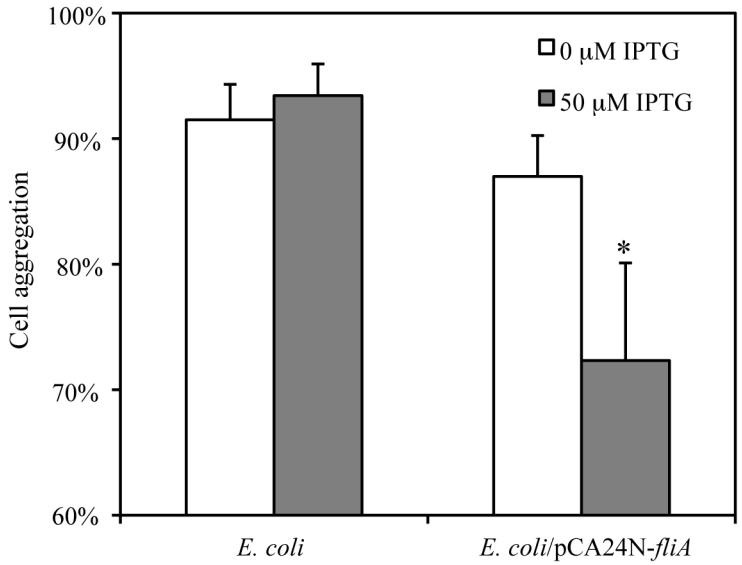

Figure 6.4 Effect of *fliA* expression on cell aggregation of *E. coli* cells

Cell aggregation of *E. coli* PHL644 and *E. coli*/pC24N-*fliA* cells cultured in 0.1×LB supplemented with 0 and 50 μM IPTG, which induced the expression of *fliA* from the plasmid pCA24N-*fliA*. Cells were re-suspended in fresh 0.1×LB for aggregation test. Cell aggregation was shown as the percentage of decreased cell density (measured in optical density) in the top layer of a column culture set static for 24 h due to formation and settling down of aggregates in gravity. The experiments were replicated independently and error bars show the standard deviation of calculated cell aggregation.

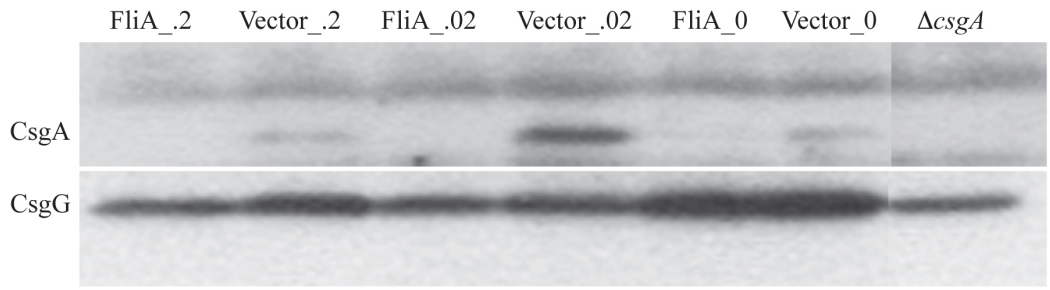

Figure 6.5 Effect of *fliA* expression on CsgA and CsgG production of *E. coli* cells

Bands of CsgA (lower band in the first row image) and CsgG on a western blot of strain *E. coli*/pTOPO-*fliA* (denoted as FliA in the figure) and *E. coli*/pTOPO (Vector) cultured in 0.1×LB broth supplemented with 0.2, 0.02 and 0% arabinose (.2, .02, and 0 in the figure) and mutant strain Δ*csgA* cultured in 0.1×LB. Cell density of tested strains in each culture was adjusted to the same optical density before loading on gels (OD600=0.2).

Table 6.1 Strains and plasmids used in this study

Strains or plasmids	Genotype	Source or reference
pCA24N-*fliA*	Plasmid of pCA24N with an IPTG inducible *fliA* gene	ASKA library [19]
pTOPO	Plasmid of pBAD-TOPO	Invitrogen pBAD-TOPO TA expression kit
pTOPO-*fliA*	Plasmid of pBAD-TOPO with an inducible *fliA* gene inserted	This study
E. coli K-12 PHL644/pMP4655	MC4100 *malA-kan ompR234,* increased curli expression, labeled with green fluorescent protein	This study
S. maltophilia/pBPF-mCherry	Environmental isolate, labeled with mCherry fluorescent protein	This study
E. coli K-12 BW25113		CGSC # 7636
E. coli/pfliA-gfp	*E. coli* K-12 MG1655 containing a plasmid pUA66 or pUA139 carrying a *gfp*-fusion with the promoter of *fliA*	*E. coli* promoter collection (PEC3877) [20]
Δ*fliA*	*fliA* knockout mutant of *E. coli* K-12 BW25113	Keio collection [21]
E. coli/pCA24N-*fliA*	*E. coli* K-12 PHL644/pMP4655 carrying the plasmid of pCA24N-*fliA*	This study
E. coli/pTOPO	*E. coli* K-12 PHL644/pMP4655 carrying the plasmid of pTOPO	This study
E. coli/pTOPO-*fliA*	*E. coli* K-12 PHL644/pMP4655 carrying the plasmid of pTOPO-*fliA*	This study

6.8 References

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Chapter 7
Conclusions and Future Directions

This dissertation research was conducted to explore the effects and to investigate genetic mechanisms of bacterial species interactions on biofilm formation. Findings in this study contribute to a better understanding of biofilm formation by one species, especially pathogenic species, with impacts from indigenous species in the same environment. The output of this study could eventually lead to development of alternative strategies for biofilm control in industrial and clinical settings. Summarized are major conclusions generated from this study, as well as suggested directions for future research.

7.1 Conclusions

The first part of this study revealed that biofilm formation by one species was affected by interactions with other species and the effect varied in different culture conditions. Biofilms were cultured in static batch systems and dynamic flow cells, simulating typical environmental conditions with standstill and running water/liquid over the surfaces where biofilms grow. *Escherichia coli* biofilm development was significantly altered in co-culture with *Stenotrophomonas maltophilia* compared to its pure culture. In flow cells, *E. coli* was promoted in biofilm formation in co-cultures, likely resulting from increased surface attachment of *E. coli* cells facilitated by *S. maltophilia* biofilm matrix. In contrast, biofilm formation by *E. coli* was inhibited (70-90% less biomass) in static batch co-culture due to nutrient competition, outcompeted surface attachment and reduced cell aggregation of *E. coli* cells. The opposite effects suggested that species interactions and their effects may change by different environmental conditions. Survival of one species, especially under the treatment of disinfectants or antibiotics, can increase by embedding in biofilm [1]. Biofilm formation and therefore the fate of one species not only depends on its interactions with co-existing species but is also influenced by local environmental conditions such as hydrodynamic parameters.

The next theme addressed in this study was the transcriptomic responses of *E. coli* to *S. maltophilia* in mixed species cultures. The purpose was to identify genes that were involved in inhibited *E. coli* biofilm formation by species interactions in static co-culture. For this purpose, an immuno-magnetic separation based method was developed to enable the application of cDNA microarray technology for the study of a mixed species community, which bypassed the current limit of this technology, which has been applied mostly to single species cultures. Genetic responses of *E. coli* to co-cultures with *S. maltophilia* were revealed, at least partially, by combining the newly developed separation method and cDNA microarray analysis. Approximately hundred genes were identified and indicated changes in metabolism, signal transduction, and cell wall component of *E. coli* in co-cultures. It is unclear at this point how all these genes are involved in species interactions. However, functional analysis of these genes showed their potential roles in several pathways, such as alternative carbohydrate uptake and ion transport, which are known to be involved in bacterial interactions by other species. Moreover, five genes (e.g., *fliA*) were confirmed to be involved in inhibiting biofilm formation in co-culture. Further characterization of these genes is warranted and will provide insights into the mechanisms of inhibiting biofilm growth by species interactions. Since the method developed in this study is not limited to separate *E. coli* for transcriptome analysis, future research can be conducted to study genetic responses of *S. maltophilia* to *E. coli*. It will result in a better understanding of responses to species interactions from both sides.

The third achievement of this study was the uncovering of one mechanism involved in the inhibition of biofilm formation of *E. coli* in mixed species cultures. Gene *fliA* is among the most highly induced genes identified by transcriptome analysis and plays an important role in flagellar and cell motility, which showed a relationship with biofilm formation in previously studies [2]. Firstly, up-regulation of *fliA* in co-culture with *S. maltophilia* was confirmed to result from direct cell contact. But cell motility was only slightly increased though *fliA* was overexpressed. On the contrary, *fliA* induction greatly repressed the production of protein CsgA, a major fiber monomer of curli that is critical for cell aggregation and biofilm formation of the *E. coli* strain used in this study. Decreased cell aggregation with *fliA* overexpression was also verified. Moreover,

knocking out gene *fliA* diminished the inhibition on *E. coli* biofilm formation in mixed species cultures. It was therefore concluded that inducing gene *fliA* to suppress curli production is at least one of the pathways in inhibiting *E. coli* biofilm formation by species interactions with *S. maltophilia.* The conclusion could have been drawn more persuasively, with evidence of less curli production of *E. coli* cells in mixed-species culture. Also left for future study is the trigger factor of *S. maltophilia* to induce *fliA* expression in *E. coli*. Cell surface components of *S. maltophilia* are suggested to be of interest, since both *fliA* induction and inhibited *E. coli* biofilm formation required cell-to-cell physical contact between the two species.

7.2 Future directions

This research examined the effect of species interactions on biofilm formation of *E. coli*, highlighted the impact of environmental conditions on the effect, and revealed in part, mechanisms of species interactions at the genetic level. The ultimate goal in practice is to control biofilm formation of incoming bacterial species, e.g. pathogens, potentially by indigenous species existing in the environment. Future research could be conducted in the following directions to reach the final goal that formed the motivation for this dissertation study. Research on other genes confirmed to be involved in biofilm formation, such as *phoH* and *yddB* can bring a more complete understanding of inhibiting *E. coli* biofilm formation. Inhibitive effect on *E. coli* biofilm formation should be tested with additional environmental strains, or a natural mixed species community. This study implies that changing hydrodynamic parameters may switch promoted to prohibited effects of species interactions on biofilm formation; therefore, effectively controlling biofilm growth of bacterial strains of interest, such as pathogens, could be achieved by altering environmental parameters. The effectiveness of this approach needs to be further tested in systems that simulate relevant environmental conditions. Uncovering an effective trigger compound for *fliA* induction, if successful in future studies, could be applied for surface coating to control biofilm growth.

Even though we have a long way to go from the basic research presented here to practical applications of effective control of biofilm formation of pathogenic species using environmental species, this study serves as a starting point of applications in

environmental health that may be similar to recently reported bacteriotherapy for human health [3].

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Appendix
Bayesian Network Expansion Identifies New ROS and Biofilm Regulators

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Bayesian Network Expansion Identifies New ROS and Biofilm Regulators

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Abstract

Signaling and regulatory pathways that guide gene expression have only been partially defined for most organisms. However, given the increasing number of microarray measurements, it may be possible to reconstruct such pathways and uncover missing connections directly from experimental data. Using a compendium of microarray gene expression data obtained from *Escherichia coli*, we constructed a series of Bayesian network models for the reactive oxygen species (ROS) pathway as defined by EcoCyc. A consensus Bayesian network model was generated using those networks sharing the top recovered score. This microarray-based network only partially agreed with the known ROS pathway curated from the literature and databases. A top network was then expanded to predict genes that could enhance the Bayesian network model using an algorithm we termed 'BN+1'. This expansion procedure predicted many stress-related genes (e.g., *dusB* and *uspE*), and their possible interactions with other ROS pathway genes. A term enrichment method discovered that biofilm-associated microarray data usually contained high expression levels of both *uspE* and *gadX*. The predicted involvement of gene *uspE* in the ROS pathway and interactions between *uspE* and *gadX* were confirmed experimentally using *E. coli* reporter strains. Genes *gadX* and *uspE* showed a feedback relationship in regulating each other's expression. Both genes were verified to regulate biofilm formation through gene knockout experiments. These data suggest that the BN+1 expansion method can faithfully uncover hidden or unknown genes for a selected pathway with significant biological roles. The presently reported BN+1 expansion method is a generalized approach applicable to the characterization and expansion of other biological pathways and living systems.

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Introduction

In this study, we explore how a biological pathway can be defined, and identify a set of methods to automatically learn a pathway from experimental data. Although many biological pathways have been described in the literature, these pathways likely represent only a small portion of the known underlying network of interactions. Recently, such pathway representations have been systematized in databases such as EcoCyc [1], RegulonDB [2], and KEGG [3]. The pathways represented in these databases are commonly used as a starting point (seed network) to analyze gene expression data and identify pathway activity using computational tools such as GSEA [4] and DAVID [5]. However, when an annotated pathway is used to analyze microarray gene expression data, the assumption is made that the ideal microarray derived network will be the same as that in the literature. This assumption may not hold since many pathways are defined based on observed protein-protein and protein-DNA interactions, metabolic fluxes, and subsets of particularly well-studied genes. Each of these factors may contribute to the substantial inconsistency between RNA-level microarray-based networks and currently defined pathways. Furthermore, the selected pathway representation may be incomplete and not include relevant regulator or effector molecules, thus necessitating computational prediction and subsequent validation. To address this issue, we introduce a method to systematically expand a pathway by identifying new genes that, from a gene expression perspective, better define the pathway itself.

Biological pathways have been constructed from the existing literature and annotation information using a wide range of methods [6,7,8,9,10,11,12,13,14]. One method of pathway reconstruction uses Bayesian networks (BNs) to learn and model relationships between variables (e.g., genes). Bayesian networks are graphical models that describe causal or apparently causal

interactions between variables. In this study, a Bayesian network is defined as a set of interactions (edges or arrows) between variables (nodes) selected from a set of known pathway genes. High scoring BN topologies are learned from data based on scoring metrics such as the BDe scoring metric introduced by Cooper et al. in 1992 [15], that incorporates the joint probabilities for variables connected to one or more other variables. In this context, the Bayesian model is a multinomial model with a uniform Dirichlet prior. Bayesian networks such as these have been used to identify relationships from gene expression data [9,16], protein-protein interactions[17,18], and the regulation of phosphorylation states [19]. Due to their flexibility, reliability, ability to model multi-variable relationships, and human interpretability, Bayesian networks are well suited for network modeling using high-throughput data such as gene expression microarrays.

Networks learned from datasets such as gene expression data can be used to expand our knowledge about a known pathway, by independently testing the effects of added genes or variables on the overall scores of the corresponding expanded networks. A general network expansion framework to predict new components of a pathway was suggested in 2001 [20]. Many of the pathway expansion methods use correlation or Boolean functions [20,21,22,23]. Compared to these methods, Bayesian network-based expansion methods provide distinct advantages, including prediction of both linear and nonlinear functions, identification of causal influences representing interactions among genes. Bayesian network-based expansion was also used for gene expression data analysis [24,25]. However, these expansion approaches are module-based methods that focus on identifying modules (or groups) of additional genes to one gene [24] or a group of genes with a fixed topology [25]. The mRNA-based networks were also merged with protein data which often do not agree with each other [25]. The topology of the biological pathways may not be consistent with networks learned from transcriptional gene expression data obtained via DNA microarray studies [21].

We hypothesize that Bayesian networks derived from microarray gene expression data are largely consistent with known pathway models and can be used as a basis to predict novel factors that influence a given pathway. In this study, the hypothesis was examined using the *Escherichia coli* reactive oxygen species (ROS) pathway. Because *E. coli* and the ROS pathway had been well studied [26,27,28,29], we were able to test the effectiveness of our network expansion algorithm and to assess the ability to reconstruct and expand an accepted pathway using microarray data. We identified many stress-related genes potentially involved in the ROS pathway and predicted their interactions with known ROS genes. Our prediction was confirmed experimentally for one example gene, *uspE*. Our single-gene expansion approach, termed 'BN+1', was successful in predicting unknown stress interactions that can be verified through experimental analysis, and could demonstrably be applied to other biological systems of interest.

Results

Below we describe the Bayesian network pathways identified from gene expression data, and the expansions to each network as predicted using the BN+1 algorithm (Figure 1).

Microarray-Based Bayesian Network Overlapped with Known ROS Pathway

Using a compendium of microarray gene expression data from the M3D database [30], networks were constructed for the 27 genes contained in the ROS pathway as defined by the EcoCyc database [1] (Figure 2). *E. coli* uses a complex detoxification pathway to protect against the oxidative stress posed by reactive oxygen species (ROS), including oxygen ions, free radicals, and peroxides [28]. The 27 genes identified in the EcoCyc ROS pathway include five ROS-processing enzymes (i.e., *katE*, *katG*, *sodA*, *sodB*, *sodC*) and 22 transcriptional factors that regulate transcription of these ROS-related enzymes. This *E. coli* expression dataset incorporates a variety of experimental conditions including time course studies, cell stress-inducing environments, over-expression, and single and double knockout strains. These conditions perturb the ROS pathway and provide a reasonable data set for the evaluation of our hypothesis. Our simulation results showed that more than one Bayesian network generated for the ROS pathway shared the same top posterior probability score. Therefore, a consensus network was derived using the 33 top

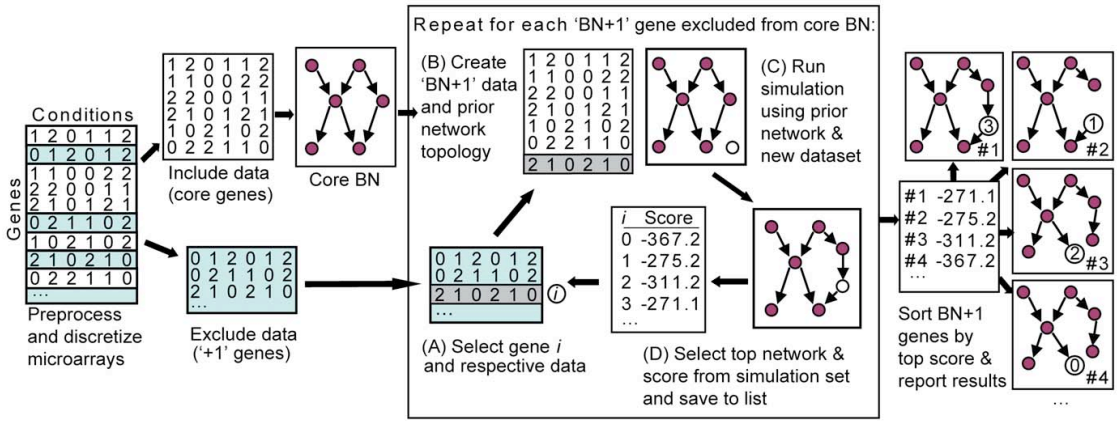

Figure 1. Schema for the BN+1 expansion algorithm. Bayesian networks are generated from discretized microarray data and ranked according to log posterior score. One of the top-scoring networks was selected as a core network for subsequent expansion. Each gene not included in the core network yet appearing in the microarray dataset was independently tested for its ability to acquire the best log posterior score versus the other tested expansion genes.
doi:10.1371/journal.pone.0009513.g001

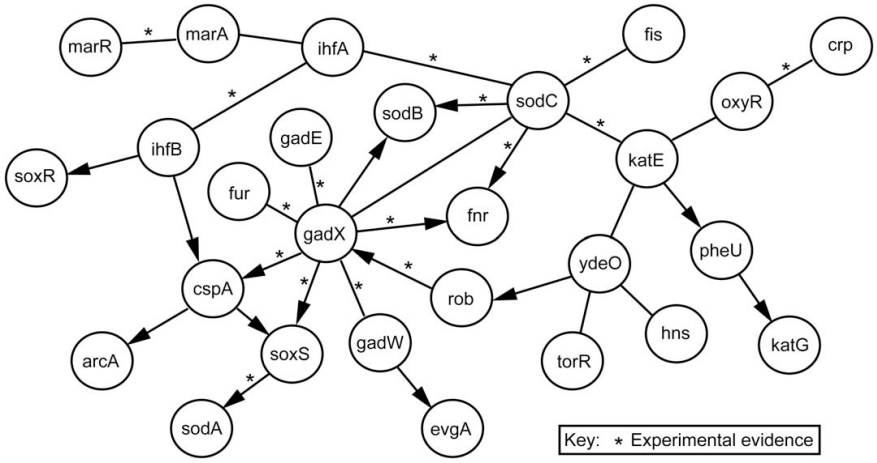

Figure 2. Consensus network for the ROS detoxification pathway based on gene expression data. Bayesian networks were generated using twenty-seven genes from the reactive oxygen species (ROS) detoxification pathway as variables or nodes and 305 gene expression microarray observations per variable. Edges which appear in the consensus and are supported by external data (e.g. EcoCyc, RegulonDB, and/or literature) are indicated (see Table S1).
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networks that shared the best identical posterior probability. The consensus network contains all 27 genes from the original ROS detoxification list in EcoCyc.

A comparison of the consensus network to EcoCyc revealed that 29% of the edges in the consensus are supported by corresponding edges in EcoCyc [1] or RegulonDB [31]. However, inclusion of literature information in the comparison revealed that approximately 42% of the edges found in the consensus network were confirmed (Table S1). The difference suggests that some new literature results have not been collected in current databases such as EcoCyc and RegulonDB.

BN+1 Pathway Expansions Predict ROS-Related Genes and Gene Interactions

An expansion algorithm termed BN+1 was developed to identify those genes that provide the best network score when added to an existing core network topology (Figure 1). This core network is a representative Bayesian network randomly selected from those top-scoring networks. Each gene not yet included in the core network is individually added to the set of variables for the Bayesian network simulation (hence Bayesian network plus one gene, or 'BN+1'). The edges in the initial core network topology are used as a 'structural prior' or starting point, and are allowed to change over the course of the BN simulations. The added node is initially disconnected from the existing core network and can become connected to other variables over the course of the simulation. Those genes which best improve the network score when added to the existing core are expected to have the most direct biological influence and/or relevance to the core network genes.

The BN+1 expansion algorithm was used to identify additional potential members of the ROS detoxification pathway. The top-ranked results from these analyses are shown in Table 1. The algorithm identifies whether a gene is strongly associated with a particular network (e.g., the ROS detoxification pathway) and which genes in the network may influence or be influenced by the newly predicted gene. The predicted influences between core genes and the top "+1" genes (including *dusB* and *uspE*) identified by BN+1 expansion are shown in Figure 3.

Expansion of the core network revealed that many top predicted genes have known relationships with ROS and stress regulation (Table 1). The tRNA-dihydrouridine synthase B gene (*dusB* or *yhdG*) was predicted to be the top-scoring BN+1 gene and to interact with *fis* and *sodC* (Figure 3A). Fis is an important regulator of oxidative stress [32]. Because all of the known enterobacterial *fis* genes are preceded by *dusB* (also called *yhdG*) within the same operon [32], it is reasonable that *dusB* is positioned as a parent of *fis* in our prediction. The gene *dusB* is highly similar to *nifR3* [32], an element of the nitrogen regulatory system in bacteria [33]. A phylogenetic anlaysis of *fis* and *dusB* indicated that both genes were acquired by a lineage ancestral to γ-proteobacteria (including *E. coli*) from the *nifR3-ntrBC* operon of an ancestral α-proteobacterial

Table 1. Top 10 genes identified by BN+1 expansion of the top Bayesian network.

Rank	Top BN+1 gene hits	Posterior BN score
1	***dusB*** (tRNA-dihydrouridine synthase B)	S = −8295.81
2	***fdhE*** (formate dehydrogenase formation protein)	S = −8298.44
3	***uspE*** (stress-induced protein);	S = −8310.63
4	***yohF*** (predicted oxidoreductase with NAD(P)-binding Rossman-fold domain)	S = −8312.24
5	***yncG*** (predicted enzyme);	S = −8313.04
6	***msyB*** (predicted protein);	S = −8318.20
7	***yedP*** (conserved protein);	S = −8320.30
8	***sra*** (30S ribosomal subunit protein S22)	S = −8323.97
9	***ydcK*** (predicted enzyme);	S = −8325.91
10	***ynhG*** (conserved protein);	S = −8326.20

Note that the numbers shown after gene names are negative logs of posterior probabilities for each top network containing the respective predicted gene.
doi:10.1371/journal.pone.0009513.t001

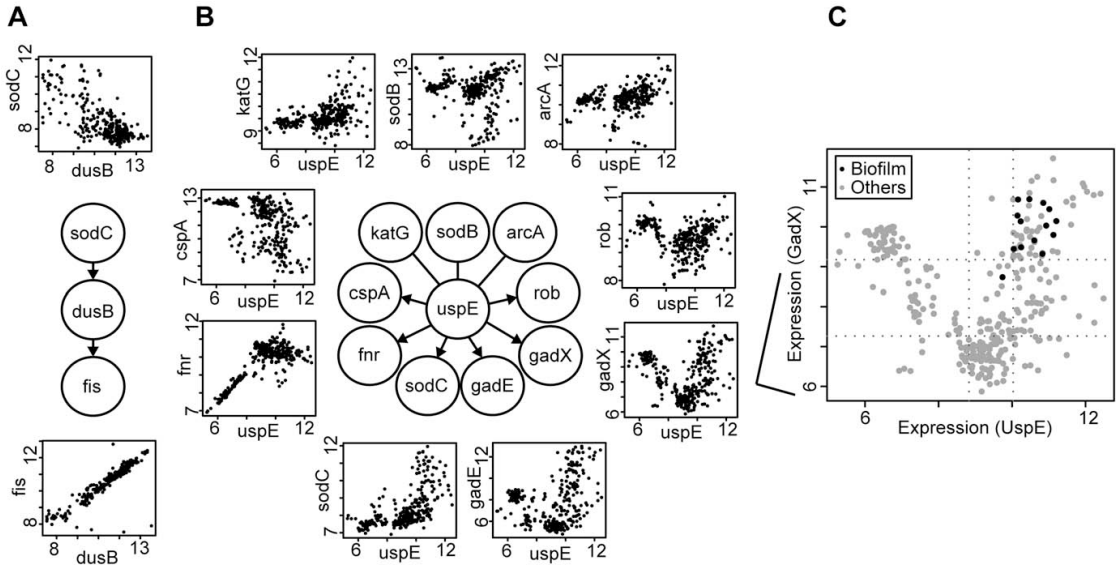

Figure 3. The genes *dusB*(A) and *uspE* (B) were the top results for the large network expansion. (C) Scatter plot for *uspE* versus *gadX* highlighting experiments with the word "biofilm" in the experiment title and/or description. High levels of *uspE* and *gadX* were observed for all conditions mapped to 'biofilm'. The dotted lines indicate boundaries for binning used in network learning. A similar profile was shown for gadE (not shown).
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lineage by lateral gene transfer [32]. Since *fis* is an important ROS regulator, it is likely that *dusB*, which was acquired together with *fis* and shares the same operon with *fis*, also plays an important role in ROS regulation. However, further experimental evidence is required to confirm the role of dusB in ROS regulation. Both *fis* and *sodC* are crucial to bacterial defense against the deleterious effects of reactive oxygen species (ROS) [34,35]. The interaction between *sodC* and *dusB* is likely important for bacterial antioxidant reactions. The second top predicted gene *fdhE* encodes an *E. coli* formate dehydrogenase accessory protein that regulates the activity of catalytic sites of aerobic formate dehydrogenases and their redox activities [36]. A third gene, the universal stress protein *uspE*, is a known major regulator of motility factors and cell aggregation under stress conditions [37]. Several other predicted enzymes (*yncG* and *ydcK*) and proteins (*msyB*) found in the BN+1 search have no currently known functions related to the ROS pathway and stress response.

Pair-wise plots of the expression of BN+1 genes versus ROS pathway genes show simple (*dusB* vs *fis*, Figure 3A) or complex relationships (*uspE* vs. *gadX*, Figure 3B–C). The plots show that the relationships between these genes may be nonlinear. For example, a "V" shaped pattern is observed between the expression profiles of *gadX* and *uspE*, where *gadX* is down-regulated at moderate levels of *uspE* and up-regulated in either increased or decreased levels of *uspE* (Figure 3C). This special non-linear gene interaction pattern was not clearly demonstrated in a traditional hierarchical clustering heatmap (Figure S1). Gene *gadX* is a transcriptional regulator of glutamic acid decarboxylase system, which enables *E. coli* to overcome acidic stress, while *uspE* is a universal stress-induced protein. A term enrichment method was generated to identify words that are preferentially grouped and reflect most significant features of the interactions between two genes (e.g., *gadX* and *uspE*) as predicted by our BN method.

Based on our term enrichment analysis of *gadX* and *uspE*, one term that clustered the data particularly well was "biofilm", which was demonstrated in the annotated scatter plot (Figure 3). High expression of *gadX* was correlated with high expression of *uspE* in biofilms. Biofilms are aggregates of microorganisms that attach to and grow on a surface in contact with liquid, such as water or media. Induced expression of stress response genes, e.g., a universal stress regulater *uspA*, was a general feature of biofilm growth [38,39]. In fact, the biofilm microarray data used in the term enrichment were obtained from two studies. One study analyzed stress-oriented gene expression profiles of *E. coli* biofilm at various time points [40]. A second biofilm microarray study examined biofilm responses to acid resistance and oxidative stress using wild type and single gene knockout mutant strains of *E. coli* [41]. Our combined analysis of microarray gene expression and term enrichment indicated that *uspE* and *gadX* were both up-regulated in many samples (chips) where 'biofilm' was mentioned in the sample title and/or description (Figure 3B–C). These suggested a potential role of the *uspE* and *gadX* in the formation of *E. coli* biofilm.

To further evaluate the interactions between *uspE* and *gadX* and their regulatory roles in ROS stress and biofilm formation, several wet-lab experiments were conducted as described below.

Confirmation of the Involvement of Gene *uspE* and *gadX* in ROS Network

Regulation of gene expression involved in the ROS network upon exposure to ROS was widely reported [26,27,28,29,34,35,37]. Hydrogen peroxide is one of the commonly used ROS. To test the involvement of *uspE* and *gadX* in the ROS network, gene expressions of *uspE* and *gadX* were monitored after exposure of two reporter strains, *E. coli* BW25113/p*gadX-gfp* and BW25113/p*uspE-gfp*, to hydrogen peroxide. GFP fluorescence

of the reporter strain indicated expression of the corresponding gene. Compared to a control not exposed to hydrogen peroxide, GFP fluorescence of both reporter strains significantly increased in exposure to both 1 mM and 10 mM hydrogen peroxide (Figure 4). This indicated that expression of *gadX* or *uspE* was up-regulated upon exposure to hydrogen peroxide. It confirmed that both genes were involved in the ROS network as predicted by our BN+1 method.

Confirmation of Interactions between *uspE* and *gadX*

To measure the interactions between gene *uspE* and *gadX*, two mutant reporter strains, Δ*uspE*/p*gadX-gfp* and Δ*gadX*/p*uspE-gfp* were generated with *gadX* and *uspE* deleted, respectively. The two mutants provide a way to monitor the effect of deleting one gene on the expression of the other gene. Specifically, GFP fluorescence of mutant reporter strains Δ*gadX*/p*uspE-gfp* and Δ*uspE*/p*gadX-gfp* were compared to fluorescence of their corresponding wild type reporter strains, BW25113/p*uspE-gfp* and BW25113/p*gadX-gfp*, respectively. The results showed that expression of gene *uspE* was significantly decreased to half level when gene *gadX* was knocked out, while *gadX* expression was significantly increased if gene *uspE* was knocked out (p-value$<$0.0001) (Figure 5). The results suggested that *gadX* induced the expression of gene *uspE*, while *uspE* may repress the expression of gene *gadX*. The fact that gene *gadX* and *uspE* influenced the expression of each other confirmed our prediction of the influences between the two genes and further refined their biological interactions.

GFP fluorescence of different *E. coli* strains (wild type or single gene knockout mutant strains) carrying reporter plasmids p*gadX-gfp* or p*uspE-gfp* indicated expression of the gene *gadX* or *uspE* in these strains, respectively, under the tested experimental conditions. The expressions of the gene *gadX* and *uspE* (GFP fluorescence of the different *E. coli* strains carrying the two reporter plasmids) under different tested conditions in the above confirmation experiments were plotted against each other

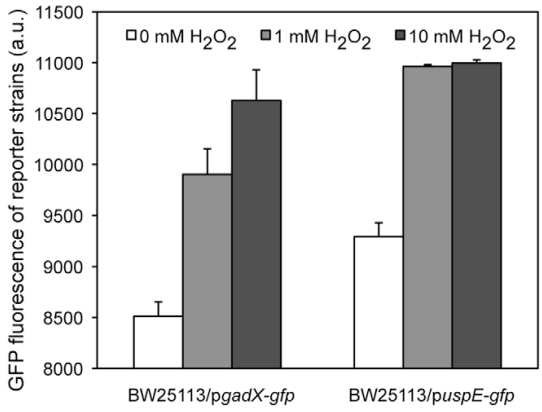

Figure 4. Expression profiles of *E. coli gadX* and *uspE* upon exposure to hydrogen peroxide. Change of GFP fluorescence of two reporter strains *E. coli* BW25113/p*gadX-gfp* and BW25113/p*uspE-gfp* upon exposure to 0 mM, 1 mM and 10 mM hydrogen peroxide for 20 min. Cells were cultured in LB broth at 30°C overnight and re-suspended in 1×PBS. Different concentration of hydrogen peroxide was added into three aliquots for 20 min before cell density (OD) and fluorescence intensity were measured. Presented GFP fluorescence for each sample was normalized to OD. Error bar indicated standard deviation from two replicated cell cultures.
doi:10.1371/journal.pone.0009513.g004

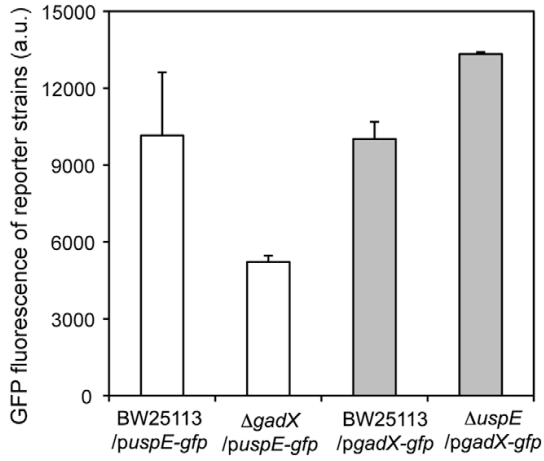

Figure 5. Analyses of the *gadX-uspE* interaction through knockout studies. GFP fluorescence of wild type *E. coli* BW25113 and single gene knockout mutant Δ*gadX* carrying the reporter plasmid p*uspE-gfp*, and wild type *E. coli* and single gene knockout mutant Δ*uspE* carrying the other reporter plasmid p*gadX-gfp*. Cells of each reporter strain were cultured in LB broth at 30°C overnight and re-suspended in 1×PBS before cell density (OD) and fluorescence intensity were measured. GFP fluorescence for each strain was normalized to the OD value. Error bars indicated standard deviations from two replicated cultures each with four replicate readings.
doi:10.1371/journal.pone.0009513.g005

(Figure 6). This plot demonstrated a roughly "V" shaped pattern similar to that shown in the plot of gene expression data pooled from microarray studies (Figure 3C).

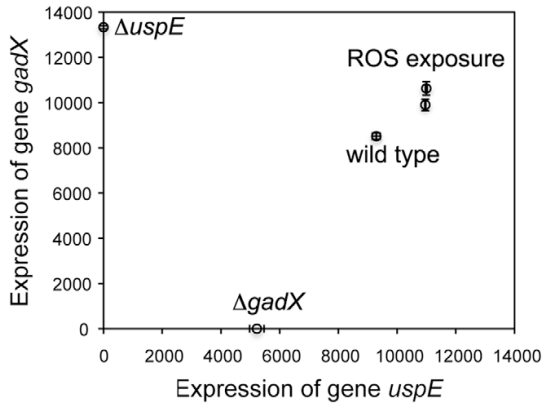

Figure 6. Summary of *gadX* and *uspE* gene expression under various experimental conditions. Plot of the expressions of *gadX* (x-axis) and *uspE* (y-axis) against each other in different strain backgrounds and tested experimental conditions. The expression of *gadX* or *uspE* was represented by the GFP fluorescence of the reporter strains carrying the respective reporter plasmids p*gadX-gfp* or p*uspE-gfp*. The strain background or experimental conditions were noted by the data. Expression of gene *uspE* or *gadX* was assumed as zero in its single gene mutant Δ*uspE* or Δ*gadX*, respectively. Wild type strain was used in the ROS exposure experiments using 1 mM and 10 mM hydrogen peroxide. Error bars indicate standard deviation from replicates.
doi:10.1371/journal.pone.0009513.g006

Confirmation of the Involvement of Gene *uspE* and *gadX* on Biofilm Formation

Biofilm cells response to a wide range of stresses [42]. Many ROS related genes have been found to be up-regulated in biofilms [38,39]. For example, *arcA* (a gene in our ROS core network) was reported to be important for competitiveness in *E. coli* biofilms [42]. Our term enrichment method identified "biofilms" as a significantly enriched term associated with the gene pair of *uspE* and *gadX*. Those microarray chips containing "biofilms" in their experimental descriptions frequently show high expressions of both *uspE* and *gadX* as demonstrated in Figure 3. To test the involvement of gene *uspE* and *gadX* in biofilm formation, initial biofilm formation (3 h attachment and growth) on glass surface by wild type *E. coli* BW25113 and single gene knockout mutants, Δ*gadX* or Δ*uspE*, was examined using confocal laser scanning microscopy (CLSM). The structure of biofilm formation was measured by a typical *en face* image of biofilms of each strain (Figure 7A–C). The extent of biofilm formation was quantified using biofilm biomass (Figure 7D). The results showed that biofilms formed by the Δ*uspE* strain contained higher biomass than biofilms formed by the wild type strain. The Δ*gadX* biofilm had similar biomass but different structures compared to biofilms by wild type *E. coli* strain. Microcolonies were observed in biofilms of wild type strain (Figure 7A), while biofilms of Δ*gadX* were mostly single layer of attached cells at this observation stage (Figure 7B). The observed difference in biofilm biomass and structure in biofilms formed by the *uspE* or *gadX* knockout mutant and wild type strain indicates that both gene *uspE* and gene *gadX* were involved in biofilm formation by *E. coli*.

In summary, the BN+1 algorithm predicted that the *uspE* gene was a new gene in the ROS network and that the *uspE* gene interacts with many ROS-related genes including *gadX*. Our further text mining analysis predicted that *gadX* and *uspE* gene may be important in biofilm formation. These three predictions were then successfully verified in experiments.

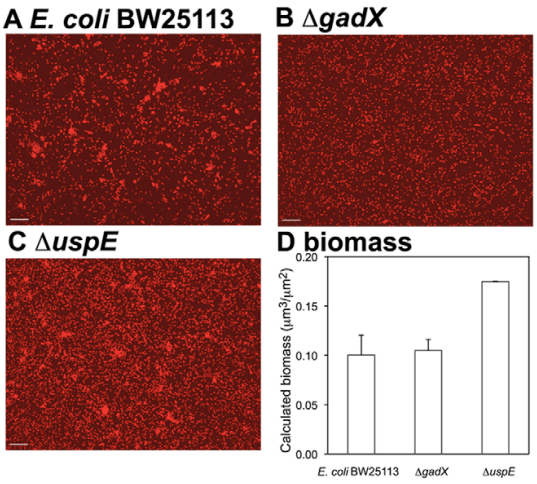

Figure 7. The effect of *gadX* and *uspE* on *E. coli* biofilm formation. Fluorescent micrograph of biofilms formed by (A) wild type *E. coli* BW25113, (B) single gene knockout mutant Δ*gadX*, and (C) single gene knockout mutant Δ*uspE*. Biomass of biofilms formed by each strain was calculated (D) using the software COMSTAT. Biofilms were formed on glass bottom of 24-well plates for 3 h after inoculation. Suspended cells were gently removed. Biofilms were gently washed with PBS twice and stained with Syto 60 for 10 min before microscopic examination. Images were taken from randomly chosen spots near the center of the well. Error bar in the calculated biomass was standard deviation from three stacks of images. Scale bar = 10 μm.
doi:10.1371/journal.pone.0009513.g007

Discussion

In this study, we addressed two questions: (1) Does a microarray-based Bayesian network reconstruction match with the known pathway from the literature and existing database? (2) Is a network expansion approach such as BN+1 useful in predicting new, biologically significant genes?

For the first question, our studies indicated that the microarray-based Bayesian network reconstruction did not always agree with the known pathway from the literature and databases. Our studies on the *E. coli* ROS pathway indicated that the network reconstructed by our Bayesian network overlaps at 29% with the known ROS pathway network in EcoCyc and RegulonDB (Table S1). A 42% agreement was achieved when more evidences from the literature search was included. Inclusion of RegulonDB and literature resources made our comparison more comprehensive. The reason for the large mismatch is probably due to the fact that microarray-based transcriptional data may not reflect the complex biological pathways which involve complex interactions of genes in the protein, RNA, and DNA levels [43]. However, the Bayesian networks built from microarray gene expression data are transcriptional regulatory models that are predicted to reflect the complex ROS pathway.

For the second question, the BN+1 expansion algorithm was found to successfully predict biologically significant genes to the ROS network that were further experimentally verified. Gene *uspE* was one of the top list genes selected by the BN+1 algorithm. Its up-regulation in response to the exposure of hydrogen peroxide suggested that this gene was probably involved in the ROS network, along with the ROS-related gene *gadX* (Figure 4). Hierarchical clustering of the *uspE* gene showed a different connectivity pattern in the dendrogram for genes than the Bayesian network, suggesting that the Bayesian network identified a non-traditional (*e.g.* nonlinear) relationship between the genes. Furthermore, the BN+1 algorithm suggested where the new genes could participate in the pathway, and in some cases the model even differentiated between the parents and children genes of a new gene (Figure 3–4). Specifically, the BN+1 algorithm found the "V" shape relationships between expressions of genes, e.g., *gadX* and *uspE*, which would not have been identified using traditional clustering approaches. The interaction between gene *gadX* and *uspE* was also confirmed experimentally. Expression of one gene was significantly affected when the other gene was knocked out from the wild type *E. coli* strain (Figure 5). Plot of the expression of *gadX* and *uspE* against each other under different tested experimental conditions showed a similar "V" shaped pattern (Figure 6), which was in agreement with the finding using the BN+1 algorithm although the expression data from the experimental study were at the translational level.

The term enrichment algorithm successfully identified experimental conditions in which genes might be involved and biologically related with each other. In this study, genes *uspE* and *gadX* were founded to be both up-regulated in the growth of biofilms. The involvement of the two genes in biofilms was confirmed by the fact that single gene knockout mutant strains Δ*gadX* and Δ*uspE* showed difference in the biofilm formation, either biomass or structures, as compared to the *E. coli* wild type strain (Figure 7). Experimental confirmation of predicted term enrichment results indicates that term enrichment algorithm is a

useful method to identify experimental conditions in which gene relationship may take place, or to propose additional areas of investigation. Performance of the term enrichment approach likely depends upon the quality of the experimental descriptions provided by researchers available from the M3D database. The approach may perform better with controlled term or concept vocabularies, or could be further tested with Gene Ontology (GO) terms and other information in future studies.

Bayesian networks can be used to expand a pathway network based on microarray gene expression data. The BN+1 method expands a top Bayesian network by adding one gene at a time and running it iteratively based on microarray gene expression data. The BN+1 expansion algorithm showed the ability to predict important factors for a pathway network from thousands of genes in a microarray study. The BN+1 approach is a generalized method to refine and expand biological pathways. Although a ROS pathway in *E. coli* was shown in this study, the BN+1 algorithm can readily be applied to other organisms, pathways, and data types. We also plan to develop a BN+1 expansion method based on dynamic Bayesian network analysis [44]. Furthermore, the term enrichment-based identification of experimental conditions in the context of binned data for BN analysis can provide beneficial information in the interpretation of predicted expansion genes.

Methods

Data Preprocessing

A compilation dataset comprising 305 gene expression microarray observations and 4,217 genes from *Escherichia coli* MG1655 was obtained from the M3D database [30]. A coefficient of variation threshold (c.v. $\geq$1.0) was used to select 4,205 genes for analysis. Twenty-seven genes were identified from the EcoCyc ROS detoxification pathway (downloaded on March 26, 2008) and matched to unique features found in 305 available gene expression microarray chips. Expression profiles for each gene were discretized using a maximum entropy approach that uses three equally-sized bins (q3 quantization).

Learning Bayesian Network Pathway Models

Given the set of 27 genes, Bayesian network analysis was used to learn the structure of the model which served as our core starting topology. To maximize the network search space, 4000 independent simulations with random starts were used to search 2.5×10^7 networks per start for a total of 1×10^{11} networks. The five top networks were saved from each run, thereby generating a final list of 20,000 top-scoring networks. These networks were used to estimate the posterior distribution. During the search, each network was scored using log of the BDe score [15,45] which is the natural log of posterior probability ($S=\ln(P(M|D))$). Here $P(M|D)$ is defined as:

$$P(M|D)\propto \prod_{i=1}^{n}\prod_{j=1}^{q_i}\frac{(r_i-1)!}{(N_{ij}+r_i-1)!}\prod_{k=1}^{r_i}N_{ijk}!,$$

where n is the number of variables, q_i is the number of parent configurations for given variable i, r_i is the arity of variable i, N_{ij} is the number of observations with selected parent configuration q_i, N_{ijk} is the number of observations of child in state k with parent configuration q_i [15]. The calculation of this score was implemented using the software package BANJO [46].

A consensus network was generated using 33 networks which shared the maximum or best log posterior score (ln(P(D|M)). Specifically, directed edges in the consensus networks represent those edges that appear with 100% frequency in one direction in all of these top networks. Undirected edges represent those edges appearing 100% of the time in both directions in all stored networks (Figure 2).

Network Expansion Using BN+1

To expand an existing network, a top network used to generate the consensus network was selected as a starting topology for the BN+1 algorithm (Figure 1). A set of 4,178 genes (4,205–27), not included in the top BN, were tested for their ability to improve score of the initial core BN when added to the initial gene set. In each iteration of the BN+1 simulation, the current BN+1 gene was added to the original data file. This was followed by a simulated annealing search of 1×10^7 networks for the top network expansion. Although the top network was selected as a starting point or seed, during the learning round all edges could be modified such that the addition of genes could change the backbone structure of the resulting model (i.e., unfixed structural prior). Genes were sorted based on their log posterior scores. BN+1 searches for each of the top 200 genes recovered from the initial top network were rerun (2.5×10^7 networks/simulation with 150 replicate simulations) to allow sufficient convergence.

All calculations, including the network expansion, were implemented in a publicly available, internally developed software program MARIMBA (http://marimba.hegroup.org/).

Term Enrichment for Identifying Relevant Experimental Observations

A term enrichment program was developed to identify which descriptive terms in the experimental conditions show significant enrichment in selected regions of the microarray data. A 'term' here is defined as any individual word appearing in the names or descriptions for each microarray sample. For two selected genes, a p-value was introduced to determine the chance of observing a selected term in a selected bin. The p-value was calculated using the Fisher's exact test for appearance of 'term' and 'non-term' data observations in a specific bin [47]. The bins used for microarray BN analysis were adopted in this term enrichment analysis. For example, the q3 quantization was used for the expression levels of *gadX* and *uspE*.

Experimental Validation of our Prediction Using Gene *uspE* as an Example

Strains and cell cultures. *E. coli* K-12 wild type strain BW25113 and single gene knockout mutant strains (Δ*uspE* and Δ*gadX*) were obtained from the KEIO collection [48]. Cell cultures were inoculated from single colonies on Luria broth (LB) agar plates, supplied with 20 μg/ml tetracycline, 30 μg/ml chloramphenicol, or 20 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) appropriately. Planktonic cell cultures were grown in LB overnight with a continue shaking (250 rpm) at 30°C.

Plasmids construction. Plasmids pUA66 or pUA139 carrying a *gfp*-fusion with the promoter of *gadX* or *uspE* were extracted from corresponding strains in the promoter library PEC3877 (Open Biosystems, Huntsville, AL) [49]. A tetracycline resistance gene (*tetR*) was cloned from pMP4655 [50] using the set of forward and backward primers, ACATGGCTCTGC-TGTAGTGA and CGACATGTCGTTTTCAGAAG respectively. Clone *tetR* was inserted in the AfeI (NEB, Ipswich, MA) digestion site of the reporter plasmids to acquire two reporter plasmids named as p*gadX-gfp* and p*uspE-gfp*. The two plasmids were individually transformed into *E. coli* BW25113 strains by electroporation (Bio-Rad, Hercules, CA). Single colonies of *E. coli*

were acquired on selective agar plates containing 20 μg/ml tetracycline. Reporter plasmids p*gadX-gfp* and p*uspE-gfp* were then extracted from single colonies of *E. coli*, and then transformed into wild type *E. coli* strains and single gene knockout mutant Δ*uspE* and Δ*gadX*, respectively, to get totally four reporter strains.

Gene expression analysis. Planktonic cultures of the four reporter strains, wild type *E. coli* BW25113/p*gadX-gfp*, BW25113/p*uspE-gfp*, Δ*uspE*/p*gadX-gfp*, and Δ*gadX*/p*uspE-gfp*, were washed and re-suspended in phosphate buffered saline (PBS). Cell growth (optical density OD at 600 nm) and fluorescence intensity of tagged GFP were measured in a plate-reader (Bio Tek, Winooski, VT). Normalized fluorescence to OD was calculated and used to indicate expression of gene *gadX* and gene *uspE* in wild type *E. coli* as well as in single gene knockout mutants. Two independent cultures were performed, each with three replicates of measurement.

Planktonic cultures of *E. coli* BW25113/p*gadX-gfp* and BW25113/p*uspE-gfp* were used to monitor expression of gene *gadX* and gene *uspE* in response to the exposure of hydrogen peroxide. Final concentration of 1 mM and 10 mM hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) was added into PBS re-suspended *E. coli* cells for 20 min. OD and GFP fluorescence intensity were measured in the plate-reader, using the same *E. coli* strains without exposure to hydrogen peroxide as controls. OD adjusted GFP fluorescence intensity was used to indicate gene expression of *gadX* or *uspE*.

GFP fluorescence of different *E. coli* strains (wild type or single gene knockout mutant strain) carrying reporter plasmids p*gadX-gfp* or p*uspE-gfp* was summarized in a plot (Figure 6), assuming that expression of the *gadX* gene and the *uspE* gene were zero in its corresponding knockout mutant, respectively.

Biofilm cultures and analysis. Planktonic cultures of wild type *E. coli* and single gene knockout mutant Δ*gadX* and Δ*uspE* were acquired from overnight cultures in 0.1×LB. Cultures were mixed with the same volume of fresh 0.1×LB before second culture at 30°C for 4 hours. New cultures were added into 24-well glass bottom plates (1 ml/well, MatTek, Ashland, MA) and kept static for three hours at room temperature to allow cells to attach onto the surface and form biofilms. Supernatant was gently removed and biofilms were washed with PBS twice. Biofilm cells were stained with 5 μm Syto 60 (Invitrogen, Carlsbad, CA) for 10 min. Biofilms were imaged randomly across the surface in the center of each well with a confocal laser scanning microscopy equipped with the software FluoView 300 (Olympus, Center Valley, PA). Biomass of biofilms was calculated using the program COMSTAT [51].

Supporting Information

Figure S1 Heatmap of gene expression profiles of all core genes and the predicted *uspE* gene. This hierarchical clustering was generated using a Manhattan distance metric and average clustering via the Heatplus module in R.
Found at: doi:10.1371/journal.pone.0009513.s001 (0.24 MB DOC)

Table S1 Database and literature evidence to support predicted Bayesian network interactions. Directed (→) and undirected (-) edges are shown for each level of consensus in the BN consensus networks.
Found at: doi:10.1371/journal.pone.0009513.s002 (0.12 MB DOC)

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Author Contributions

Conceived and designed the experiments: APH DD PJW CX YH. Performed the experiments: APH DD. Analyzed the data: APH DD PJW CX YH. Contributed reagents/materials/analysis tools: APH DD ZX PJW CX YH. Wrote the paper: APH DD PJW CX YH.

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